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Influence of β-catenin inhibition on monocyte migration towards glioblastoma stem cells

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„Walk on, walk on
With hope in your heart
And you'll never walk alone."

Richard Rodgers & Oscar Hammerstein II (1945)

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Zusammenfassung

Das Glioblastom (GBM) ist der häufigste bösartige hirneigene Tumor des Erwachsenen. Innerhalb dessen Tumormasse sowie in der unmittelbaren Umgebung – der sogenannten Tumormikroumgebung (TME)- befinden sich heterogene, miteinander interagierende Zellpopulationen. Eine dieser Subpopulationen sind Glioblastom-Stammzellen (GSZ), welche für Therapieresistenz und Rezidive des GBMs verantwortlich gemacht werden. Aktuelle Forschungsansätze zeigen, dass GSZ mit Immunzellen interagieren und die Differenzierung der Immunzellen z.B. zu tumor-assoziierten Makrophagen (TAM) fördern. TAM verhindern eine gegen den Tumor gerichtete Immunantwort und unterstützen gleichzeitig Tumorwachstum und -infiltration. Der Wnt/β-catenin Signalweg fördert hierbei Stammzelleigenschaften der GSZ und wurde zuletzt auch mit Immunevasion von Tumoren in Verbindung gebracht. Um die Mechanismen der Wnt/β-catenin assoziierten Immunevasion besser verstehen zu können, soll in dieser Arbeit der Einfluss von β-catenin in GSZ auf die Chemotaxis von Monozyten, den Vorläuferzellen von TAM, untersucht werden. Nach genetischer Suppression von β-catenin zeigte sich eine Reduktion der Migration CD14-positiver Monozyten in Richtung GSZ sowie eine reduzierte CCL2-Sekretion *in vitro*. Durch Hinzufügen von rekombinantem CCL2 konnte die Migration der Monozyten wiederhergestellt werden. Genetische CCL2-Suppression ergab eine vergleichbare Reduktion der Migration CD14-positiver Monozyten wie die β-catenin Suppression. Die genetische CCL2-Inhibition veränderte darüber hinaus Expression und Aktivität von β-catenin, was auf Interaktionsmechanismen zwischen den beiden Proteinen hindeutet. Die pharmakologische β-catenin Suppression mit dem Inhibitor Methyl 3-{(4methylphenyl)sulfonylamino}benzoate (MSAB) führte zu einer verminderten Wnt/β-catenin-Aktivität, einer verminderten Klonogenität sowie einer erhöhten Apoptoserate in Glioblastomzellen, zeigte jedoch andersartige Auswirkungen auf deren CCL2-Sekretion als die genetische β-catenin Suppression.

Zusammenfassend zeigt die Arbeit, dass sowohl β-catenin als auch CCL2 in GSZ *in vitro* an der Chemotaxis von Monozyten beteiligt sind. Einige Ergebnisse geben dabei Hinweise auf einen gegenseitigen Einfluss von β-catenin und CCL2 in GSZ. Pharmakologische β-catenin Suppression mit MSAB reduziert die Wnt/β-catenin-Aktivität in GSZ, führt zu deren Apoptose und einer veränderten CCL2-Sekretion.

Für diese Arbeit lagen gültige Ethikvoten (5841-R,2019-484) vor.

Summary

Glioblastoma (GBM) is the most common malignant primary brain tumor. GBM tumor mass and so-called tumor microenvironment (TME) are characterized by heterogeneous and interacting cell populations. Glioma stem cells (GSCs) are a subpopulation that is responsible for therapy resistance and recurrence of GBM by mediating tumor initiation and self-renewal. In previous studies, GSCs have been shown to influence immune cells to differentiate into a tumor-supportive phenotype, e.g., in tumor-associated macrophages (TAMs). TAMs impair an effective anti-tumor immune response and support tumor invasion. Stem cell pathways including the (Wnt)/β-catenin pathway are active in GSCs. The Wnt/β-catenin pathway promotes stemness, but also immune escape mechanisms. By genetical and pharmacological inhibition this work aims to characterize the role of β-catenin in chemotaxis of monocytes – the precursor of TAMs. shβ-catenin decreases monocyte migration towards GBM cells as well as CCL2 secretion of GBM cells *in vitro.* The addition of recombinant CCL2 to the supernatant of shβ-catenin cells restores peripheral blood-derived mononuclear cell (PBMC) migration, while genetic CCL2 inhibition reduces monocyte migration towards GBM cells similarly to β-catenin inhibition. Furthermore, CCL2 inhibition influences the expression and activity of β-catenin, which indicates interaction mechanisms between β-catenin and CCL2. Pharmacological inhibition of β-catenin by Methyl 3-{(4methylphenyl)sulfonylamino}benzoate (MSAB) decreases Wnt/β-catenin activity and clonogenicity while increasing apoptosis in GBM cells. In comparison to the genetic β-catenin suppression, treatment with MSAB shows different effects on CCL2 secretion in tested GBM cell lines.

This study shows that both β-catenin and CCL2 in GBM cells promote the chemotaxis of monocytes towards GSCs *in vitro*. Some results imply interdependence between β-catenin and CCL2 which may contribute to the maintenance of GSCs and their crosstalk with TAMs. Pharmacological treatment with MSAB decreases Wnt/β-catenin activity, increases apoptosis, and alters CCL2 expression.

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Abkürzungsverzeichnis

Akt: synonym to Protein kinase B (PKB) Ak strain transforming

APC: adenomatous polyposis coli protein

APCs: antigen-presenting cells

Axin2: axis inhibition protein 2

BATF3: basic leucine zipper transcription factor 3

BBB: blood-brain barrier

c-Myc: C-myelocytomatosis oncogene product

CBP: CREB-binding protein

CCL2: CC-chemokine ligand 2

CCR2: C-C chemokine receptor type 2

CD4: cluster of differentiation 4

CD8: cluster of differentiation 8

CD14: cluster of differentiation 14

CD27: cluster of differentiation 27

CD28: cluster of differentiation 28

CD44: cluster of differentiation 44

CD133: cluster of differentiation 133

CD137: cluster of differentiation 137

CD278: cluster of differentiation 278

CNS: central nervous system

CTLA-4: cytotoxic T-lymphocyte-associated Protein 4

CTNNB1: catenin beta 1

DC: dendritic cell

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

EGFR: epidermal growth factor receptor

EGFRvIII: epidermal growth factor receptor variant III

EMT: epithelial-mesenchymal transition

ERK: extracellular signal-regulated kinase

FZD: frizzled

GBM: glioblastoma multiforme

GSC: glioma stem cell

GSK3β: glycogen synthase kinase 3β

GSZ: Glioblastomstammzelle

h: hour(s)

HCC: hepatocellular carcinoma

ICI: immune checkpoint inhibition

IDH: isocitrate dehydrogenase

IDO-1: indoleamine 2,3-dioxygenase 1

IL-1β: interleukin 1β

IL-10: interleukin 10

L: liter

LAG-3: lymphocyte-activation gene 3

LRP5: lipoprotein receptor-related protein 5

LRP6: lipoprotein receptor-related protein 6

m: milli or meter

M: molar

M1: classically activated macrophages

M2: alternatively activated macrophages

MAPK: mitogen-activated protein kinase

MDSC: myeloid-derived suppressor cell

MGMT: O-6-methylguanine-DNA methyltransferase

MHC I: major histocompatibility complex class I

MMP2: matrix metalloproteinase 2

MMP9: matrix metalloproteinase 9

MRI: magnetic resonance imaging

mRNA: messenger RNA

MSAB: methyl 3- [(4-methyl phenyl)sulfonyl]amino-benzoate

mTOR: mammalian Target of Rapamycin

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Musashi-1: RNA-binding protein Musashi homolog 1

Nestin: acronym for neuroepithelial stem cell protein

NF1: neurofibromin 1

NFAT: nuclear factor of activated T-cells

NLK: nemo-like kinase

NK: natural killer

Oct4: octamer-binding transcription factor 4

PBMC: peripheral blood-derived mononuclear cell

PD-1: programmed cell death protein 1

PD-L1: programmed cell death protein ligand 1

PI3K: phosphoinositide-3-kinase

POSTN: periostin

PORCN: Porcupine

SHH: sonic hedgehog

shRNA: small hairpin ribonucleic acid

SNAIL: zinc finger protein SNAI1

SOX2: (sex determining region Y)-box 2

RAS: rat sarcoma

RAF: rapidly accelerated fibrosarcoma

rGBM: recurrent glioblastoma multiforme

RNA: ribonucleic acid

RT-qPCR: real-time quantitative PCR

TAM: tumor-associated macrophage

TCF/LEF: T cell factor/lymphoid enhancer factor family

TCF4: transcription factor 4

TERT: telomerase reverse transcriptase

TF: transcription factor

TIM-3: T-cell immunoglobulin and mucin domain-containing protein 3

TME: tumor microenvironment

TMZ: temozolomide

Tregs: regulatory T cells

TTF: tumor-treating fields

VEGF: vascular epidermal growth factor

WHO: World Health Organisation

WISP-1: wnt1 inducible signaling pathway protein 1

WNT: wingless and Int-1

ZEB: zinc finger E-box binding homeobox

ZEB1: zinc finger E-box binding homeobox 1

β-catenin: beta-catenin

µ: micro

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1 Introduction

1.1 Glioblastoma (GBM)

1.1.1 Classification of gliomas

Gliomas are primary tumors of the Central Nervous System (CNS), which are derived from neuroglial stem or progenitor cells. According to the World Health Organization (WHO), classification is primarily based on histologic characteristics and molecular biomarkers [2]. More and more molecular biomarkers are arising from novel diagnostic technologies and the classification is constantly developing [2]. Currently, Gliomas are grouped into 6 families: Adult-type diffuse gliomas, pediatric-type diffuse low-grade gliomas, pediatric-type diffuse high-grade gliomas, circumscribed astrocytic gliomas, glioneuronal and neuronal tumors, as well as ependymal tumors [2].

Furthermore, CNS tumors are divided into four grades (CNS WHO Grade 1-4), which are predominantly based on histological parameters including growth pattern, tissue type, cellularity, the existence of necrosis or microvascular proliferation, pleomorphism, and mitotic activity [2]. For example, grade I gliomas are characterized by a circumscribed growth pattern, while grade 4 gliomas grow infiltrative with high mitotic activity and show necrosis as well as pathologic microvascular proliferation [2]. Recently, specific molecular markers were also included in the grading process to improve prognostic information [2].

1.1.2 Characteristics of glioblastoma

In former classifications, the term ‘Glioblastoma’ was used for Glioblastoma, isocitrate dehydrogenase (IDH)-wildtype (primary or *de-novo* Glioblastoma) as well as for IDH-mutant astrocytic glioma with WHO grade IV (secondary Glioblastoma) [3]. According to the 2021 WHO Classification of Tumors of the Central Nervous System and different from former classifications the term ‘Glioblastoma’ is now only used for Glioblastoma, IDH-wildtype [2]. Glioblastoma, IDH-wildtype (GBM) belongs to the family of adult-type diffuse gliomas and is diagnosed if an IDH-wildtype, astrocytic glioma in adults has at least one of the following criteria: microvascular proliferation, necrosis, telomerase

reverse transcriptase (TERT) promoter mutation, epidermal growth factor receptor (EGFR) gene amplification and/or +7/-10 chromosome copy number changes [2]. Interestingly, due to the knowledge of prognostic relevant molecular markers, GBM is always assigned to CNS WHO grade 4, even though histopathological high-grade features can be missing [2].

GBM is the most common malignant primary brain tumor with a proportion of around 50% of all malignant primary CNS tumors [4]. It occurs mostly in elderly patients with a median age of 65 years at diagnosis [5]. The gold standard diagnostic for GBM is structural magnet resonance imaging (MRI) with gadolinium as the contrast agent. Most GBMs are found in cerebral hemispheres, especially in frontal or temporal lobes, and present as highly infiltrative tumor masses with a marginal uptake of contrast agent [6]. 35% of the tumors are multifocal at the time of diagnosis [7].

1.1.3 Standard treatment of glioblastoma

After diagnosis, glioblastoma is treated with maximal safe resection [8,9] followed by chemoradiation, including six weeks of simultaneous radiotherapy and oral temozolomide (TMZ) treatment, as well as six cycles of adjuvant TMZ [10]. Regarding TMZ treatment methylation status of the O-6-methylguanine-DNA methyltransferase (MGMT) gene is prognostically relevant [11]. TMZ works as an alkylating antineoplastic agent. Removing alkyl groups of the O-(6)-position of guanine, MGMT is a deoxyribonucleic acid (DNA) repair enzyme that can reverse the effect of TMZ [11]. MGMT promoter methylation leads to decreased enzyme activity of tumor cells and therefore to a better therapy response to TMZ [11,12].

A newer therapeutic approach includes a mild electrical field that pulses through the skin of the scalp and disturbs cell division and is called Tumor-treating fields (TTF) [13]. As a complementary treatment to adjuvant TMZ, it showed a survival benefit compared to patients who only received TMZ [13]. TTF should be worn at least 18 hours per day and are used until the second progression or for a maximum of 24 months [13]. For recurrent Glioblastoma (rGBM) Lomustine and vascular epidermal growth factor (VEGF) inhibitor Bevacizumab are commonly used agents [14-18]. Despite multimodal treatment, the median survival is only 16-18 months [5]. Therefore, new therapeutic targets are needed in GBM.

1.1.4 Immunotherapeutic approaches in glioblastoma

Immunotherapy is based on overcoming immunosuppressive mechanisms created by tumor cells and regaining anti-tumor immune response. Anti-tumor immunity is mainly characterized by the following key steps [19]: mutations lead to the appearance of modified proteins – so-called neoantigens or tumor antigens – which are recognized by antigen-presenting cells (APCs) [20]. APCs include dendritic cells, macrophages/microglia, and B cells. These cells take up antigens, break them into peptides, and present them – bound to major histocompatibility complex class I (MHC I) – to T cells [21]. T cells are activated by the interaction between MHC and T cell receptors. This process is influenced by stimulating or inhibiting co-receptors. Cluster of differentiation receptor 8 (CD8) is a co-receptor that is expressed on the surface of cytotoxic T cells, but also on natural killer cells (NK cells) and dendritic cells (DCs). Binding of compatible T cell and CD8 receptors of a cytotoxic T cell with the MHC I complex of an APC activates the cytotoxic T cell following a cascade to kill the antigen-expressing tumor cell [22]. Stimulating co-receptors including CD27, CD28, CD137 or CD278 induce T cell differentiation and proliferation, while inhibitory co-receptors programmed cell death protein 1 (PD-1), programmed cell death ligand 1 (PD-L1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) suppress these processes [23,24]. The latter are physiologically expressed on the surface of many tissue cells to prevent autoimmunity [25]. Both co-stimulatory and co-inhibitory receptors are also called ‘immune checkpoints’ and are targeted by immunotherapy [26].

Immunotherapeutic approaches including immune checkpoint inhibition and tumor vaccines have already been studied in GBM [27]. Especially in recent years, immune checkpoint inhibition gained attention showing promising results in melanoma or lung cancer [28-31]. Immune checkpoint inhibitors are monoclonal antibodies that target inhibitory immune checkpoints e.g., PD-1/PD-L1 and CTLA-4 [32]. Tumor cells upregulate these proteins and prevent anti-tumor immune response by impairing effector T cell activation [32]. Multiple immune checkpoints such as PD-1, TIM-3, lymphocyte-activation gene 3 (LAG-3), and CTLA-4 are expressed in GBM and its microenvironment [33-38]. However, treatment with immune checkpoint inhibitors (ICI) in GBM did not show a significant benefit in overall survival *in vivo* compared to standard treatment (anti-

PD-1 vs. TMZ and anti-PD-1 vs. bevacizumab) [39-41]. Further studies including combination treatments are ongoing [42].

Besides immune checkpoints, another limitation of the anti-cancer immune response is the low immunogenicity of tumor antigens [43]. It has been shown, that only 1.2% of tumor antigens induce a spontaneous T cell response in patients with melanoma, gastrointestinal, lung, and ovarian cancers [43]. Therefore, another strategy is based on vaccines and aims to prime the adaptive immune system by presenting one or multiple specific tumor antigens [44]. For GBM a vaccine against epidermal growth factor receptor variant III (EGFRvIII) was evaluated and showed a significant effect on median overall survival in recurrent GBM in combination with bevacizumab [45]. Here it must be mentioned, that not all GBMs express EGFRvIII [46], meaning the vaccine is only beneficial for a certain percentage of GBM patients. Therefore, multi-peptide vaccines targeting multiple antigens, partly based on dendritic cells, are tested in Phase III studies, but have not shown promising results yet [47-49].

In summary, previous immunotherapeutic approaches in GBM have not yet stood up to the promising results in other cancers [27,42]. Therefore, further studies are needed to understand better which mechanisms are involved in GBM immune escape and its resistance to immunotherapy.

1.1.5 Challenges for immunotherapy in glioblastoma

Because of the blood-brain barrier (BBB), the brain was first seen as an immune-privileged organ in which peripheral immune cells are mostly excluded. In recent years it was discovered that in inflamed status peripheral blood cells are recruited into the brain [50-52]. Importantly it could be seen that GBM is susceptible to the immune system and infiltrated by various immune cells [53-55]. Still, failure of immunotherapy is imminent in GBM and is thought to be multifactorial [27,42].

Firstly, GBM has been shown to have a high intertumoral and intratumoral heterogenicity with distinct and dynamic TMEs [56-59]. In 2010 Verhaak et al. introduced four clinically relevant subtypes of GBM by analyzing genomic alterations: classical, mesenchymal, proneural, and neural [56]. Later the neural subtype was identified as neural lineage contamination [60]. Transcriptomic and epigenetic profiling revealed six subgroups, partly confirming, and partly further dividing the previously claimed

subgroups [61]. Interestingly, different subtypes and subtype-specific alterations were associated with different immune cell landscapes [55,57]. For example, the mesenchymal subtype, which is characterized by the deactivation of the neurofibromin 1 gene (NF1), showed a higher infiltration of tumor-associated macrophages (TAMs) and a lower activation of NK cells [60]. In classic subtype chromosome 7 amplification, chromosome 10 loss and high-level EGFR amplification are frequently found [56]. Here, the dendritic cell activation gene signature was higher than in the other subtypes [60].

Moreover, single-cell analysis displayed various cell types within glioblastoma tumor mass [59]. After treatment, resistant subpopulations arise and cause tumor maintenance and recurrence [57,62]. In recent studies it was demonstrated, that 55% of recurrent GBM samples and 90% of druggable targets are significantly different from the primary tumor [57,62]. Especially, so-called 'glioma stem cells' (GCSs) are seen as tumor-initiating cells, which are responsible for therapy resistance [63]. In summary, intertumoral and intratumoral heterogenicity in combination with the various corresponding immune microenvironments make immunotherapy in GBM more challenging [42].

Secondly, while a high mutational load of a tumor correlates positively with the success of immunotherapy, most GBMs are characterized by a low mutational burden [64-66]. A high number of mutations leads to the generation of neo-antigens, which can be detected by the immune system [65]. However, only 3.5% of GBM show a high tumor mutational load [64]. Indeed, it was shown that GBM patients with more mutations show a better response to anti-PD-1 therapy [67,68].

Besides those characteristics, there are indications of systemic and local immunosuppression in GBM patients [42]. Systemic immunosuppression is based on higher sequestration of T cells in the bone marrow as well as iatrogenic factors such as radiotherapy, chemotherapy, and the use of corticosteroids [69-71]. Local immunosuppression mechanisms seem to be more complex and encompass the interplay between tumor cells and immune cells in the tumor microenvironment (TME) [57]. Tumor cells – including glioma cells – impair anti-tumor immunity by secretion of cytokines and other immune regulatory proteins [38,72-77]. For example, they exclude $CD8^+$ T cells which are crucial in anti-tumor immunity and associated with a better prognosis in most cancers [75,78] from the tumor mass [38]. On the one hand, $CD8^+$ T cells even show a higher rate of apoptosis around tumor cells [38,79]. On the other hand,

tumor cells recruit immune cells to the tumor site and impact their differentiation: in the TME APCs differentiate into more immunosuppressive phenotypes with a higher expression of inhibitory immune checkpoints resulting in an impaired antigen-presentation and decreased effector function [74,80-84]. As a consequence, immune cell subpopulations like regulatory T cells (Tregs) [85-87], TAMs [57,88], and myeloid-derived suppressor cells (MDSCs) [89-92] are frequently found in tumors including GBM. Infiltration of these cells is associated with a worse prognosis and response to immunotherapy [87,93-99].

However, reciprocal interaction mechanisms between the different GBM subpopulations and immune cells are complex and still incompletely understood [57]. The following work focuses on GSCs and TAMs.

1.2 Glioma stem cells and tumor-associated macrophages in GBM

1.2.1 Glioma stem cells

GBM has been shown to be a highly heterogeneous tumor with a variety of different cell populations within the tumor mass [59]. Glioma stem cells (GSCs) were identified as a tumor-initiating cell population distinguished by a high capacity of self-renewal [100,101]. They are identified by the expression of stem cell markers like CD133, (sex determining region Y)-box 2 (SOX2), Nestin, Nanog, CD44, octamer-binding transcription factor 4 (Oct4), and RNA-binding protein Musashi homolog 1 (Musashi-1) [102]. In Xenograft models GSCs were able to initiate a whole tumor growth with the development of heterogenous subpopulations [59]. GSCs are found in the perivascular niche of glioblastoma and secrete vascular growth factors as well as immunosuppressive cytokines [102-104]. Showing resistance to chemo- and radiotherapy GSCs are seen to contribute to the failure of current therapeutic strategies and the high recurrence rate of GBM [63,101,105-111]. Therapy resistance and stem cell characteristics are conveyed by aberrant reactivation of embryonic stem cell pathways like Wingless (Wnt)-, Notch- or Sonic hedgehog (SHH)- pathways [106,108,111,112].

In both embryonic development and carcinogenesis, these pathways are linked to a mechanism called epithelial-mesenchymal transition (EMT) [113]: activated transcription factors of the zinc finger E-box binding homeobox (ZEB)-, TWIST, and snail family zinc finger (SNAI)-family induce cells to lose intercellular junctions and gain

mesenchymal characteristics followed by the ability to migrate into other tissues [114-117]. Therefore, the EMT process is responsible for invasiveness and metastasis in cancer [118]. Initially discovered in epithelial tumors, EMT-like mechanisms were also found in GBM [118].

1.2.2 Tumor-associated macrophages in glioblastoma

TAMs are the dominant immune cell population in GBM [53,57,88,119-121]. TAMs have been characterized as a heterogenous immune cell population which is partly derived from blood circulating $CD14^+$ monocytes [81,88,89] and is mainly found around the perivascular niche [122]. Glioma cells recruit monocytes to the tumor site by secretion of different cytokines [104,123]. Recruited monocytes cross the blood-brain barrier and infiltrate into the tumor, followed by differentiation into macrophages [124]. Depending on activation stimuli macrophages differentiate into pro-inflammatory phenotypes or immunosuppressive/-regulative phenotypes [88,125,126]. Subpopulations of macrophages differ in receptor expression, effector function, cytokine, and chemokine production [88,126,127]. Around tumor cells, macrophages predominately show immunosuppressive and tumor-promoting behavior and are thus called 'tumor-associated macrophages' [104]. TAMs promote glioma growth, angiogenesis, and invasion by secretion of tumor-promoting factors like interleukin 10 (IL-10) or VEGF [128]. Simultaneously, they secrete anti-inflammatory cytokines and impair anti-tumor immune response by prohibiting effector T cell infiltration and priming [84,124,129]. Therefore, increased TAM infiltration in GBM correlates positively with glioma grade and negatively with prognosis [94-97]. Interestingly, a higher TAM infiltration was found in relapses after irradiation, chemotherapy, and antiangiogenic therapy, indicating involvement in therapy resistance [97,130-133]. TAMs are associated with a lower sensitivity to immune checkpoint inhibition [134], making them an interesting target to improve the efficiency of immunotherapy.

1.2.3 Interaction between glioma stem cells and tumor-associated macrophages

TAMS and GSCs are both found in the perivascular niche and hypoxic regions of GBM [102,122,135]. Recent studies highlighted different ways of reciprocal interaction and support between these subpopulations: GSCs actively recruit monocyte-derived TAMs to the tumor site e.g., by secretion of Periostin (POSTN) and Osteopontin signaling [136,137]. Furthermore, GSCs influence macrophage differentiation, leading to

decreased phagocytosis function and an increased secretion of immunosuppressive, tumor-promoting cytokines on the one hand [104,135]. On the other hand, TAMs contribute to the maintenance and invasiveness of GSCs [138]. Despite growing knowledge, the mechanisms of interaction between GSCs and TAMs remain incompletely understood. This thesis concentrates on the Wnt signaling pathway in GSCs and its influence on monocyte recruitment.

1.3 Wnt signaling pathway

1.3.1 Canonical and non-canonical Wnt signaling

Wingless (Wnt) signaling designates an evolutionary conserved pathway, which is physiologically active in embryogenesis. The name 'Wnt' is a fusion of integration 1 (int1) and wingless. Wnt signaling is divided into the canonical and non-canonical cascades. It is involved tissue development including cell polarity, cell migration, and organogenesis. In adult humans, the pathway is mostly inactive but involved in bone homeostasis [139]. However, aberrant signaling plays a role in tumor initiation, growth, and metastasis [140].

The canonical Wnt signaling pathway is activated by Wnt proteins binding to receptors of the frizzled family (FZD) and corresponding co-receptors lipoprotein receptor-related protein (LRP) 5 or 6. Activation prevents axis inhibitor protein (Axin), glycogen synthase kinase 3β (GSK-3β), and adenomatous-polyposis-coli (APC) protein from forming a destruction complex and degrading β-catenin. As a key player in canonical Wnt cascade, β-catenin accumulates in the cytoplasm, enters the cell's nucleus and binds to transcription factors from the T cell factor/lymphoid enhancer factor (TCF/LEF)-family [139].

The non-canonical Wnt signaling is less well understood. After induction by Wnt proteins 4, 5a, 5b, 6, 7a, 7b, and 11 intracellular calcium is released and increases levels of nemo-like kinase (NLK) as well as nuclear factor of activated T-cells (NFAT). NLK inhibits the β-catenin/TCF transition complex, while NFAT is shown to be important in immune response mechanisms [141].

1.3.2 Targeting Wnt pathway in glioblastoma

Aberrant Wnt/β-catenin signaling has been found in several cancers [140]. While for example, in colorectal cancer mutations in the APC gene and CTNNB1 (β-catenin gene)

mainly lead to constitutive activation of Wnt signaling, in GBM it is mainly based on epigenetically silenced Wnt antagonists [142]. The canonical Wnt pathway is associated with poorer prognosis in GBM and is active in GSCs [143-152]. As mentioned above, Wnt/β-catenin activation increases the transcription of EMT processing genes in GSCs. One target gene of the pathway is zinc finger E-box binding homeobox 1 (*ZEB1*) which directly correlates positively with GSC markers and promotes invasiveness as well as chemoresistance of GBM [151]. Interestingly, β-catenin also increases the expression of the DNA repair enzyme MGMT which contributes to the chemoresistance of GBM [153]. The activity of non-canonical Wnt signaling is not as well understood. Wnt-5a and -5b, common activators of the non-canonical pathway, are overexpressed in GBM [154,155]. Wnt-5a expression promotes proliferation and tumor formation capacity [154,155].

Pharmacological inhibition of Wnt signaling was tested with different compounds – mostly targeting canonical signaling – and showed effectiveness in decreasing glioma proliferation *in vitro* and *in vivo* [156]. LGK974, which suppresses the palmitoylation of Wnt proteins by porcupine inhibition and therefore both Wnt pathways, reduces resistance against temozolomide in GBM *in vitro*, indicating a synergistic effect of Wnt inhibition and current standard therapy [112,150].

In this study, Methyl 3-{(4methylphenyl)sulfonylamino}benzoate (MSAB) was tested in three GBM cell lines [1]. MSAB is a small molecule inhibitor, which directly binds to β-catenin and leads to its proteasomal degradation [157]. Consequently, it leads to a suppression of canonical Wnt signaling. Hwang et al. demonstrated that MSAB is effective in different Wnt-dependent cancer cell lines and reduces tumor growth in mouse xenograft models while not affecting Wnt-independent cancer cells [157].

1.3.3 Wnt signaling in cancer immunity

Besides admitting stem cell features and processing EMT-like transition, Wnt/β-catenin is increasingly associated with cancer immune evasion [158]. As mentioned above, the success of immunotherapy is predicted by the infiltration of $CD8^+$ effector T cells [159]. Initially discovered in melanoma, β-catenin expression negatively correlates with infiltration of $CD8^+$ T cells in different cancer entities including GBM [160,161]. Active β-catenin signaling was even shown to cause resistance to immune checkpoint inhibitors [160,162]. These discoveries led to a new perception of Wnt/β-catenin as an important driver of tumor immunity.

Recent studies discovered, that Wnt/β-catenin disrupts the recruitment of APCs, basic leucine zipper transcription factor 3 (BATF3)-dependent dendritic cells respectively [163]. Tumor cells also activate β-catenin in APCs including dendritic cells followed by increased activity of immunosuppressive indoleamine 2,3-dioxygenase 1 (IDO1) [164]. The result is higher recruitment of regulatory T-cells and inhibition of cytotoxic T-cell activity [165]. Further, Wnt/β-catenin is associated with an upregulation of PD-L1 in tumor cells, including GBM [166]. As mentioned above PD-L1 is an immune checkpoint that impairs the activation, expansion, and effector functions of antigen-specific $CD8^+$ T cells and induces regulatory T cells [33]. Intrinsic Wnt activation in T cells inhibits T cell differentiation towards effector $CD8^+$ T cells [167].

In contrast, in MDSC and NK cells, β-catenin shows contrary effects: Canonical Wnt-signaling decreases accumulation and infiltration of immunosuppressive MDSCs into the tumor [168]. In NK cells it promotes maturation and effector function [169].

Therefore, Wnt/β-catenin involvement in cancer immunity is complex and context-dependent [158].

1.3.4 Wnt signaling in the interaction of tumor cells and TAMs

This study focused on monocyte-derived immune cells and their interaction with tumor cells in dependence on Wnt/β-catenin signaling. Some studies indicated, that Wnt signaling plays a role in the crosstalk between these cell populations [170].

Tumor-conditioned media and co-culturing of tumor cells and macrophages drives M2-polarization in TAMs [104]. Secretion of Wnt target Wnt1 inducible signaling pathway protein 1 (WISP-1), cytokine IL-10, and other Wnt ligands promote the survival of tumor-supportive TAMs [171-175].

Wnt activation in TAMs increases their tumor-supporting functions including migration and invasion [176,177]. In hepatocellular carcinoma, TAM intrinsic Wnt/β-catenin inhibits $CD8^+$ T cell proliferation [173]. In lung cancer mouse models Wnt inhibition both reduced tumor growth and induced a shift of TAMs to a M1-like phenotype [175].

TAMs secrete Wnt ligands as well as tumor-promoting factors leading to cancer growth and progression, indicating reciprocal maintenance of Wnt activation: For example, TAM-derived Wnt2b supports the EMT process in hepatocellular carcinoma (HCC) cells [174], while Wnt5b inhibition in ovarian cancer stem cells decreases their chemotherapy resistance and migration *in vitro* and *in vivo* [178]. In addition, interleukin 1β (IL-1β)

secreted by macrophages leads to a stabilization of β-catenin in colon cancer cells, suggesting an interaction between immunogenic cytokines and β-catenin pathway [179]. In summary, Wnt signaling both in immune cells and cancer cells plays a critical role in anti-cancer immunity [158]. Here, especially Wnt-driven TAM-cancer cell interaction seems to be an appealing target for both impairing tumor growth and supporting anti-tumor immunity.

1.4 Objective

Despite multimodal treatment and extensive research, GBM remains a tumor with a poor prognosis [5]. Immunotherapy failed to achieve the hoped impact due to unfavorable characteristics of GBM and incomplete knowledge about the immunologic processes within GBM tumor mass and tumor microenvironment [42].

Both, the Wnt/β-catenin pathway and TAM infiltration in GBM are associated with a poorer prognosis and an impaired T cell response in GBM [95,143,145,161]. It has been shown that the Wnt/β-catenin pathway is active in GSCs [151] and TAMs are enriched around GSCs [135]. However, the underlying mechanisms remain only partially understood. In this context, it is crucial to investigate the involvement of the Wnt/β-catenin pathway in the recruitment of TAMs. The correlation of Wnt/β-catenin activity with TAM infiltration could be used as a prognostic marker and a combined therapeutic target in GBM.

This thesis aims to investigate if Wnt/β-catenin signaling is involved in the recruitment of $CD14^+$ monocyte-derived TAMs.

This work is based on one publication using genetic and pharmacological β-catenin suppression to address these questions. It further investigates the participation of CCL2 in this context and evaluates the small molecule MSAB as a potential pharmacological β-catenin inhibitor [1].

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Crosstalk between β-Catenin and CCL2 Drives Migration of Monocytes towards Glioblastoma Cells

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Abstract: Isocitrate dehydrogenase (IDH)-wildtype glioblastoma (GBM) is a fast growing and highly heterogeneous tumor, often characterized by the presence of glioblastoma stem cells (GSCs). The plasticity of GSCs results in therapy resistance and impairs anti-tumor immune response by influencing immune cells in the tumor microenvironment (TME). Previously, β-catenin was associated with stemness in GBM as well as with immune escape mechanisms. Here, we investigated the effect of β-catenin on attracting monocytes towards GBM cells. In addition, we evaluated whether CCL2 is involved in β-catenin crosstalk between monocytes and tumor cells. Our analysis revealed that shRNA targeting β-catenin in GBMs reduces monocytes attraction and impacts CCL2 secretion. The addition of recombinant CCL2 restores peripheral blood mononuclear cells (PBMC) migration towards medium (TCM) conditioned by shβ-catenin GBM cells. CCL2 knockdown in GBM cells shows similar effects and reduces monocyte migration to a similar extent as β-catenin knockdown. When investigating the effect of CCL2 on β-catenin activity, we found that CCL2 modulates components of the Wnt/β-catenin pathway and alters the clonogenicity of GBM cells. In addition, the pharmacological β-catenin inhibitor MSAB reduces active β-catenin, downregulates the expression of associated genes and alters CCL2 secretion. Taken together, we showed that β-catenin plays an important role in attracting monocytes towards GBM cells in vitro. We hypothesize that the interactions between β-catenin and CCL2 contribute to maintenance of GSCs via modulating immune cell interaction and promoting GBM growth and recurrence.

Keywords: glioblastoma; GSCs; β-catenin; Wnt; CCL2; monocytes; immune evasion; MSAB

1. Introduction

Despite multimodal treatment including supramarginal resection, radiotherapy and chemotherapy isocitrate dehydrogenase (IDH)-wildtype glioblastoma (GBM), the most common malignant primary brain tumor has a median survival of less than two years [1,2]. Therapy resistance and recurrence tendency in GBM have been attributed to the presen

of GSCs [3–10], which promote cancer initiation and progression [11–16]. GSCs have been characterized by special metabolic [17] and immunologic behavior [18]. An important intracellular pathway inducing stem cell properties in GBM is the canonical Wnt/β-catenin signaling [19–28], a highly conserved pathway, which directs cell development, migration and polarity during embryonic development and in carcinogenesis. In GBM, Wnt/β-catenin drives glioblastoma cell survival, migration and maintenance of GSCs [19–28]. Furthermore, recent evidence indicates that β-catenin—the pathway's key protein—leads to the exclusion of immune cells from the tumor environment of different cancer types, thus preventing anti-tumor immunity [29–33].

Monocyte-derived tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSC) are commonly found in the GBM tumor mass, with TAMs being the dominant GBM infiltrating immune cell population [34–39]. Functionally, TAMs promote tumor growth and metastasis by impairing the anti-tumor immune response [40–46], among others, by producing chemokines such as C–C motif chemokine ligand 2 (CCL2) [47–49]. CCL2 possesses both tumor-inhibitory and tumor-promoting effects, depending on the interaction between cancer and host cells [47,50–53]. Originally known as a monocyte chemoattractant and pro-inflammatory protein, it has also been shown to drive angiogenesis and metastasis in the TME of different cancer types including GBM [47,54–61].

A positive correlation of GSCs and TAMs has been observed in GBM, suggesting an important role of GSCs in TAM-recruitment [62,63]. A recent study demonstrated that a β-catenin-CCL2 feedback loop mediates crosstalk between cancer cells and macrophages in breast cancer stem cells [64]. Given that the Wnt/β-catenin pathway is active in GSCs [19–28], we investigated the effect of β-catenin signaling on monocyte migration and potential involvement of CCL2 in β-catenin-dependent cross-talk between monocytes and GBM cells. Because of the numerous molecular and genomic differences between adult and pediatric GBM, we used two adult and one pediatric cell lines for compari- son. Furthermore, we performed pharmacological targeting of β-catenin with the small molecule inhibitor (Methyl 3-{(4methylphenyl)sulfonylamino}benzoate, MSAB) [65] to evaluate Wnt/β-catenin inhibition and apoptosis-inducing ability in this context.

2. Material and Methods

2.1. Cell Culture and MSAB Treatment

We used three GBM cell lines: GBM1 (adult male, classical subtype, MGMT methylated, IDH wild type) was generously provided by A. Vescovi (Milan, Italy) JHH520 (adult female, mesenchymal subtype, MGMT methylated, IDH wild type) was provided by G. Riggins (Johns Hopkins Hospital Baltimore, Baltimore, MD, USA) and SF188 (8-year-old male, MGMT unmethylated, IDH wild type) was provided by C. Eberhart (Johns Hopkins Hospital Baltimore, Baltimore, MD, USA). HEK293T were purchased from American Tissue Culture Collection (Manassas, VA, USA). All GBM cell lines were cultivated in neurosphere medium containing 70% DMEM w/o pyruvate and 30% Ham's F12 nutrient mix (both Gibco BRL, Eggenstein, Germany), supplemented with 2% serum free B27 (Gibco BRL), 20 ng/mL bovine fibroblast growth factor, 20 ng/mL human epidermal growth factor (both Peprotech, Rocky Hill, NJ, USA), 5 µg/mL heparin (Sigma-Aldrich, St. Louis, MO, USA) and 1% Anti-Anti Penicillin-Streptomycin Fungizone® mixture (Gibco). HEK293T cells were cultivated in DMEM with pyruvate (Gibco) supplemented with 10% Fetal Calf Serum (FCS; Biochrome, MD, USA) and 1% Anti-Anti Penicillin Streptomycin Fungizone® mixture (Gibco). Cells were cultured under standard conditions (37 °C, 5% CO_2), and routinely tested for mycoplasma contamination using the PCR-based Mycoplasma Test Kit I/C from Promokine (Heidelberg, Germany) MSAB (Sigma-Aldrich) was diluted in DMSO (Sigma-Aldrich) and stored at −20 °C. For apoptosis assay, immunoblotting and ELISA, cells were cultured for 24 h under general cell culture conditions in the presence of various concentrations of MSAB diluted in neurosphere medium.

2.2. Generation of Lentiviral Particles

The third-generation lentiviral packaging system was used for the generation of lentiviral particles, as previously described [21]. HEK293T cells were transfected with the lentiviral vector of choice and three different packaging plasmids (pMDLgpRRE, pRSVREV and pMD2VSVG) using FuGENE® HD transfection reagent (Promega, Madison, WI, USA). Supernatants containing the viral particles were collected after 48, 72 and 96 h post transfection and passed through a 0.45-micron filter before being concentrated using polyethylene glycol and sodium chloride (NaCl). Viral particles were stored at −80 °C. The CCL2 knockdown was achieved by cloning shRNA into the pLKO.1 TRC vector (Addgene plasmid, Addgene, Cambridge, MA, USA) [66]. GBM cell lines (GBM1, JHH520, SF188) were transduced with lentiviral particles containing shβ-catenin/shCCL2 plasmids. Transfected cells were selected using 2 µg/mL puromycin (Sigma-Aldrich). The proliferation and migration assay were performed after stable conditions, and sufficient cell numbers were achieved between eleven to thirteen days after transduction.

2.3. Cell Viability and Cell Death Assays

GBM cell lines were seeded in triplicates on 96-well-plates at a density of 1.5×10^4 cells/mL and cultivated in 100 µL neurosphere medium for a total of six days. The viability was assessed using the Thiazolyl Blue Tetrazolium Bromide assay (MTT, Sigma-Aldrich), according to the manufacturer's instructions. Absorbance was measured at 570 nm (reference 650 nm) using a Paradigm™ multiplate reader (Beckman Coulter, Brea, CA, USA). Cells were treated with MSAB at 1, 1.5 and 2.25 µM diluted in neurosphere medium for 24 h. Control cells were treated with vehicle (DMSO) only. To assess cell death after MSAB treatment, the MUSE Annexin V & Dead Cell Kit (Merck Millipore, Burlington, MA, USA) was used and cells were prepared according to manufacturer's instructions. The analysis was performed using the MUSE cell analyzer (Merck Millipore).

2.4. Clonogenicity Assay

To assess the clonogenic capacity of cell lines, we performed colony formation assay in soft agarose, as described previously [22]. Briefly, six-well plates were coated with a bottom layer consisting of 1.5 mL of 1% agarose (Life Technologies, Carlsbad, CA, USA) and neurosphere medium. A 2 mL layer consisting of 0.6% agarose containing 5×10^3 cells/well was coated on top and it was covered with additional medium (2 mL). After 3 weeks of incubation under standard cell culture conditions, 1 mg/mL 4-Nitro tetrazolium chloride (NBT) solution (Sigma-Aldrich) was added to stain the colonies overnight at 37 °C. The experiments were quantified using Clono Counter software [67].

2.5. Quantitative Real Time PCR (RT qPCR)

RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentrations were measured photometrically using the Nanodrop2000 spectrometer (Thermo Scientific, Waltham, MA, USA). Two micrograms of RNA were utilized to synthesize complementary cDNA single strands using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and random hexameric primers. Quantitative real time PCR was performed using advanced SYBR Green Supermix (BioRad, Hercules, CA, USA), 10 ng of cDNA and 10 pmol of each primer. Data were analyzed in a CFX Connect Thermocycler (BioRad). Relative expression levels of genes were normalized to the endogenous housekeeping gene β-actin. The Primer sequences can be found in Supplementary Table S1.

2.6. Whole Genome Transcriptome Analysis

Whole genome transcriptome analysis (3′mRNA sequencing) was performed at the NGS Core Facility (Bonn, Germany). The R package Deseq2 was applied to identify differentially expressed genes in the control cells versus the β-catenin knockdown cells. The

R package clusterProfiler was used to view these differentially expressed genes enriched in the KEGG pathways.

2.7. Western Blotting

Cells were lysed in ice-cold RIPA buffer and protein concentrations were determined using the DC Protein Assay Kit (BioRad) following manufacturer's instructions. Incubation with primary antibodies against active β-catenin (1:1000, BD Sciences, Franklin Lakes, NJ, USA) and β-actin or GAPDH (1:5000, Thermo Fisher) was performed overnight at 4 °C on a 3D-shaker in 5% BSA (VWR Life Science, Radnor, PA, USA) in TBST. As secondary antibodies, we used goat-anti-rabbit antibody IRDye800CW (1:10,000, LI-COR #926-32211, Lincoln, NE, USA) and goat-anti-mouse antibody IRDye680RD (1:10,000, LI-COR #926-68070) diluted in blocking solution and incubated for 1 h at room temperature. Signal detection was performed on a luminescence-based system in a LI-COR Odyssey CLx Imager (LI-COR). Luminescence values for active β-catenin were normalized to the corresponding GAPDH or β-actin values.

2.8. ELISA

Cells were seeded at a density of 5×10^5 cells/mL in neurosphere medium. Supernatants were collected after 24 h and passed through a 0.2 µM micron filter before being stored at −20 °C until needed. ELISA was performed using Human MCP-1 (CCL2) Standard ABTS ELISA Development Kit (Peprotech) following manufacturer's instructions. ABTS Liquid Substrate (Sigma-Aldrich) was utilized and color development was measured at 405 nm with wavelength correction set at 650 nm using Paradigm™ multiplate reader (Beckman Coulter, Brea, CA, USA). Measured values were compared to obtain standard curves and normalized to total protein concentrations determined by DC Protein Assay Kit (BioRad).

2.9. PBMC Migration Assay

The migration assay was performed using 6.5 mm diameter Transwell cell culture inserts (5 µm pore size; Costar, Washington, DC, USA; REF3421). Human PBMCs isolated from the blood of healthy donors were isolated by Ficoll density centrifugation, washed, counted and re-suspended in serum-free RPMI medium in the upper chamber of the filter (1×10^6 cells in 500 µL). In the lower chamber, 800 µL of tumor-conditioned media was added. Cells were left to migrate for 4 h at 37 °C. Afterwards, cells remaining on the upper surface of the filter were removed with a cotton swab. Cells that migrated to the lower chamber were collected. Live cells were re-suspended in trypan blue and counted using a hemocytometer.

In an additional experiment, PBMCs were harvested and stained with fluorescently labeled antibodies to assess monocyte migration. For this, PBMCs were stained with Zombie-Yellow Live/Dead stain, incubated with CD16/CD32 Fc blocking antibody and stained with an antibody against CD14 (FITC, 1:100 dilution). Samples were run on a Beckman Coulter Gallios flow cytometer and analyzed using the Kaluza 2.1 software.

2.10. Luciferase Reporter Assay

To detect canonical Wnt pathway activity, we stably transfected GBM cells with a reporter construct containing seven TCF-binding sites followed by a firefly luciferase cassette as described previously [22]. Transfected cells were selected using 2µg/mL puromycin (Sigma-Aldrich). For each measurement, cells were harvested and washed in PBS. Cells were treated with MSAB at 10 µM diluted in neurosphere medium for 24 h. Control cells were treated with vehicle (DMSO) only. Cells were prepared according to manufacturer's protocol (ThermoFisher Scientific, Madison, WI, USA). Luminescence readout was performed at 490 nm emission wavelength on Paradigm™ multiplate reader (Beckman Coulter, Brea, CA, USA) and normalized to ß-galactosidase activity.

2.11. Statistical Analyses

All data were obtained from three independent replicates and are shown as mean ± SD. Statistical significance was calculated using an unpaired student's *t* test using *GraphPad Prism software*, version 8.0 (GraphPad Software, San Diego, CA, USA). Differences were considered significant for a *p* value of $p < 0.05$.

3. Results

3.1. β-Catenin Expression by GBM Cells Impacts Monocyte Migration and CCL2 Secretion

To identify the impact of β-catenin in glioma cells on immune cell migration, we first established a β-catenin knockdown using small hairpin RNA (shRNA) interference in three GBM cell lines (GBM1, JHH520 and SF188).

Reduced expression of *CTNNB1* (gene encoding β-catenin) was confirmed by qPCR (Figure 1A) and at protein level by Western blot (Figure 1B). Reduced β-catenin expression decreased proliferation of both GBM1 (Day 4: $p = 0.049$; Day 6: $p = 0.015$) and JHH520 cells (Day 2: $p = 0.007$; Day 4: $p = 0.016$) (Supplementary Figure S1A). We performed comprehensive gene expression analyses to determine the impact of β-catenin knockdown on GBM cell lines (Supplementary Figure S2). The analysis revealed several genes with altered expression (GBM1: n = 39 upregulated, n = 87 downregulated; JHH: n = 65 upregulated, n = 178 downregulated; SF188: n = 79 upregulated, n = 89 downregulated) (Supplementary Figure S2A,B). The results of KEGG pathway analysis showed that the differentially expressed genes (DEGs) were highly associated with signaling pathways ranging from N-glycan biosynthesis to metabolism and carcinogenesis in these cell lines (Supplementary Figure S2C). As expected, the expression of a large number of genes closely associated with the Wnt/β-catenin pathway was affected by knockdown of β-catenin (Supplementary Figure S3).

To investigate the effect of β-catenin in GBM cells on immune cell migration, we performed a Boyden chamber assay in which PBMCs migrated through a porous membrane towards media conditioned by GBM cells. Tumor conditioned media (TCM) derived from β-catenin knockdown GBM cells significantly reduced the number of migrated PBMCs compared to TCM derived from control cells (Supplementary Figure S1C). We next investigated the effect of β-catenin expression on monocyte migration. Similarly, TCMs collected from GBM cells with reduced β-catenin expression decreased monocyte migration significantly in JHH520 ($p = 0.046$) and SF188 ($p = 0.012$), whereas the reduction observed for shβ-catenin GBM1 cells did not reach statistical significance (Figure 1C).

CCL2 is a strong chemoattractant for monocytes and has already been associated with β-catenin expression [47,68–75]. We therefore investigated how β-catenin expression affects CCL2 production. β-catenin suppression significantly decreased CCL2 gene expression in GBM1 ($p = 0.0119$) and JHH520 ($p = 0.0432$) cells (Supplementary Figure S1B) as well as CCL2 protein levels in TCM of GBM1 ($p \leq 0.001$) and JHH520 cells ($p = 0.0001$) (Figure 1D). SF188 showed a similar, yet not statistically significant reduction in CCL2 protein, but not in the mRNA level.

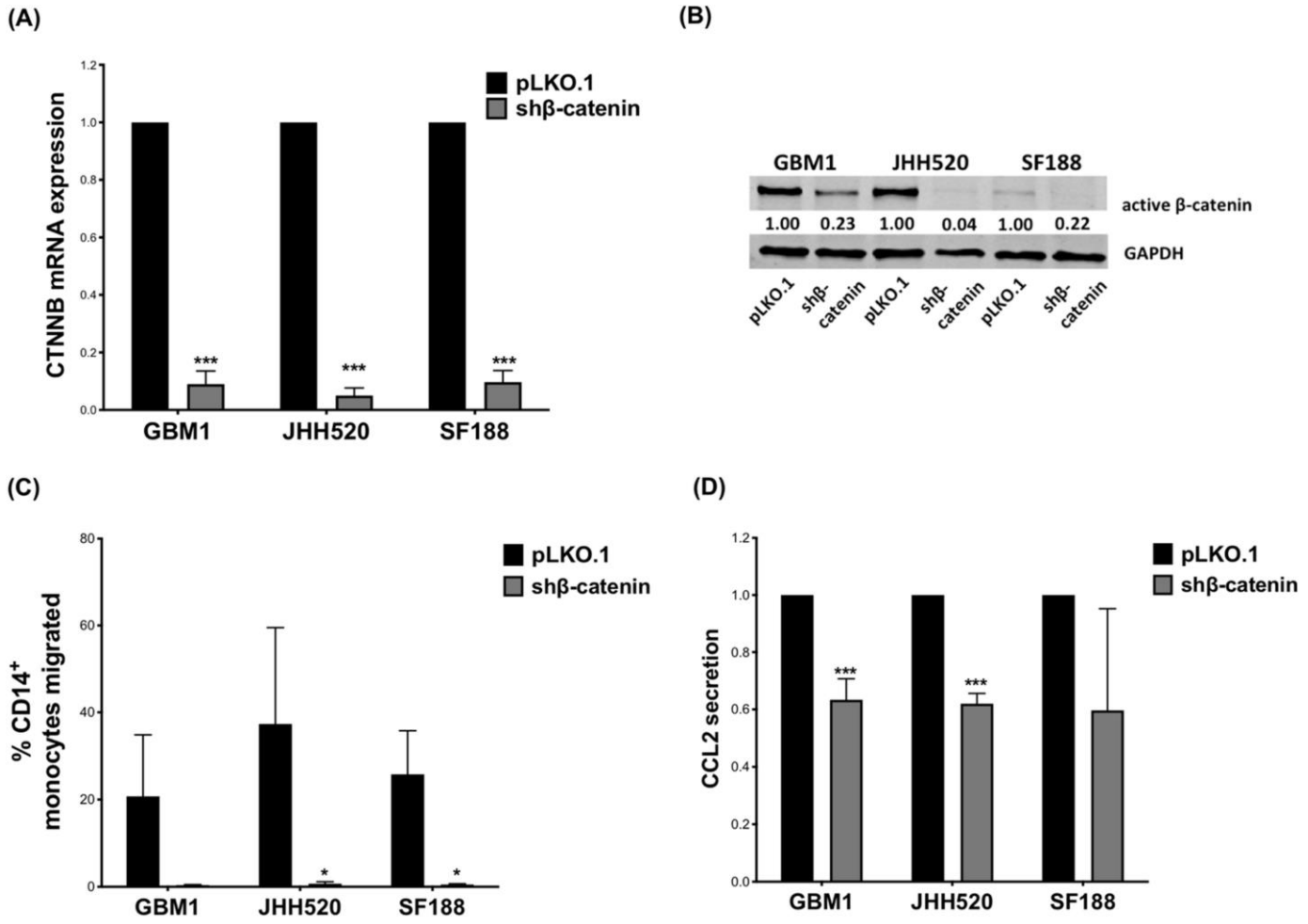

Figure 1. β-catenin knockdown in GBM cells reduces migration of $CD14^+$ monocytes in vitro: (**A**) GBM cell lines (GBM1, JHH520, SF188) were transduced with lentiviral particles containing shβ-catenin plasmids and knockdown efficiency (relative mRNA expression) was confirmed using RT-qPCR and (**B**) Western blotting. (**C**) $CD14^+$ monocyte migration towards TCM of β-catenin knockdown cells was decreased compared to migration towards TCM of control (pLKO.1) cells. **(D)** CCL2 levels in TCM of shβ-catenin GBM cells were measured after 24 h incubation by ELISA and compared to control cells (pLKO.1). The relative CCL2 secretion data are presented as mean ± SD (n = 3). Statistical significance was calculated with unpaired *t*-test. * $p \leq 0.05$ *** $p \leq 0.001$.

3.2. Recombinant CCL2 Restored PBMC Migration in shβ-Catenin TCM and CCL2 Knockdown Reduced Monocyte Migration

To confirm that the observed decrease in CCL2 expression was responsible for the reduced migration of monocytes towards TCM from GBM cells with reduced β-catenin expression, we added recombinant CCL2 (100 ng/mL) to β-catenin knockdown TCMs, which restored PBMC migration (Figure 2A).

To investigate if a similar effect on monocyte migration can be observed in CCL2 knockdown GBM cells, we used shRNA to suppress CCL2 production. We confirmed reduced CCL2 gene expression (Figure 2B) and CCL2 secretion (Figure 2C) compared to control cells (pLKO.1). A slight decrease in proliferation was observed in CCL2 knockdown GBM cells, particularly of JHH520 cells (Day 2 $p = 0.033$, Day 4 $p = 0.0171$) (Supplementary Figure S4A–C). Indeed, similar to β-catenin knockdown, CCL2 knockdown significantly reduced $CD14^+$-monocyte migration compared to control (Figure 2D, JHH520 ($p = 0.049$) and SF188 ($p = 0.013$). Again, in GBM1 the decrease did not reach statistical significance.

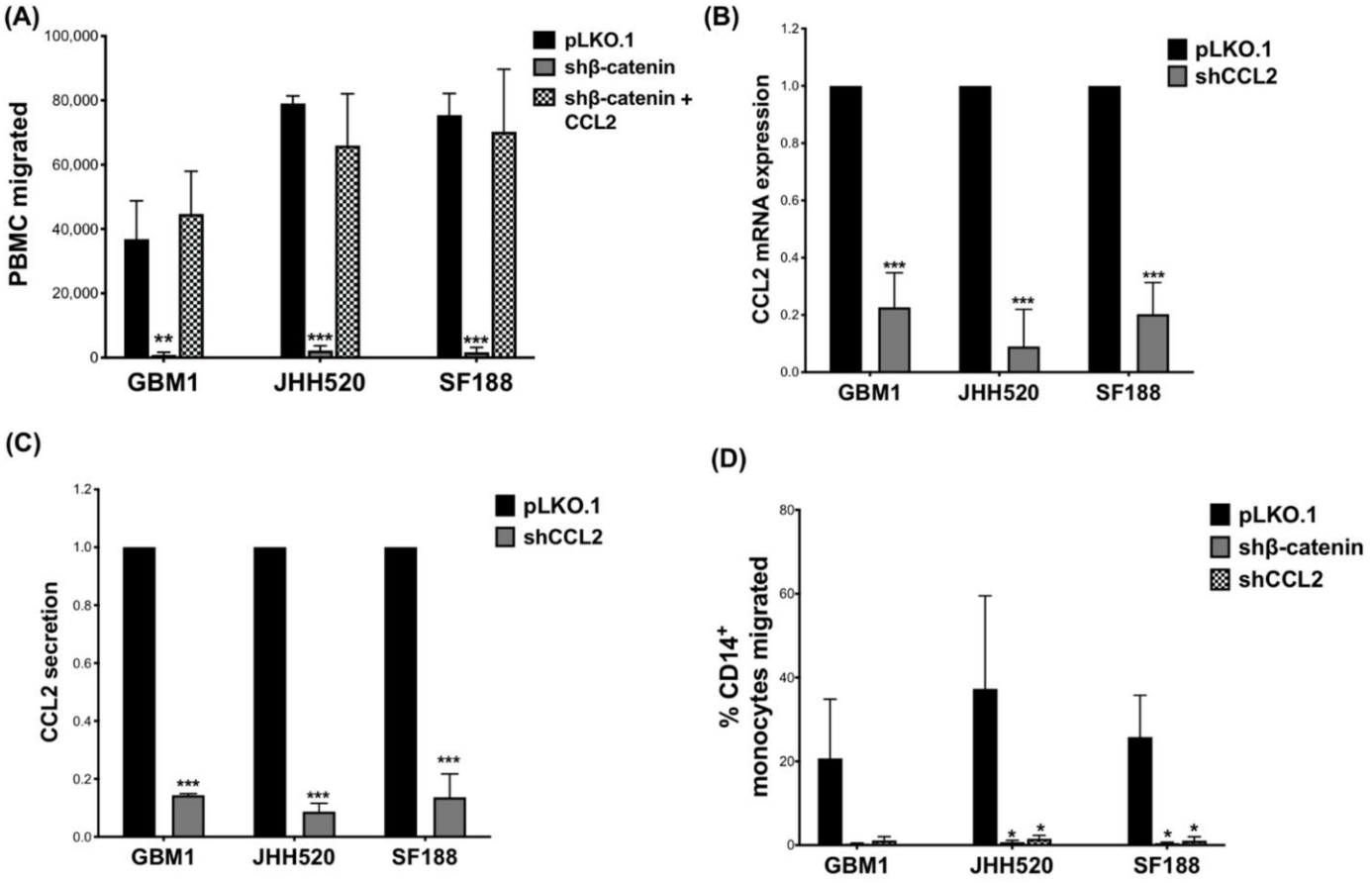

Figure 2. Recombinant CCL2 restored PBMC migration in shβ-catenin TCM and CCL2 knockdown reduced monocyte migration: (**A**) Recombinant CCL2 (100 ng/mL) was added to the TCM of β-catenin knockdown cells and restored PBMC-attracting ability. GBM cell lines were transduced with lentiviral particles containing shCCL2 plasmids and knockdown efficiency (relative mRNA expression and relative CCL2 secretion) was confirmed using (**B**) RT-qPCR and (**C**) ELISA, respectively. (**D**) $CD14^+$-monocyte migration was decreased after treatment with TCM of shCCL2 knockdown cells compared to treatment with TCM of control (pLKO.1) cells. Data are presented as mean ± SD (n = 3). Statistical significance was calculated with unpaired *t*-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.3. CCL2 Modulates Components of the Wnt/β-Catenin Pathway and Alters Clonogenicity of GBM Cells

To determine the effects of CCL2 on β-catenin activity, we further analyzed the phenotype of CCL2 suppressed GBM cells. We investigated the expression of β-catenin target (*AXIN2*, *MYC*) and further EMT–related genes (*ZEB1*, *SNAI1* and *SNAI2*). Following CCL2 knockdown, *CTNNB1* mRNA expression was upregulated, though not statistically significant in GBM1 and SF188. *SNAI2* expression was significantly reduced in all cell lines (Figure 3A). Gene expression of *AXIN2*, *MYC*, *ZEB1* and *SNAI1* was significantly different in JHH520 cells, but this could not be confirmed in GBM1 and SF188 cells (Figure 3A). Western blot analysis revealed significantly reduced β-catenin protein levels in GBM1 ($p = 0.0013$) and JHH520 ($p = 0.0013$) compared to the control cells (pLKO.1). In SF188 cells, CCL2 suppression significantly increased β-catenin protein levels (Figure 3B, $p = 0.0003$).

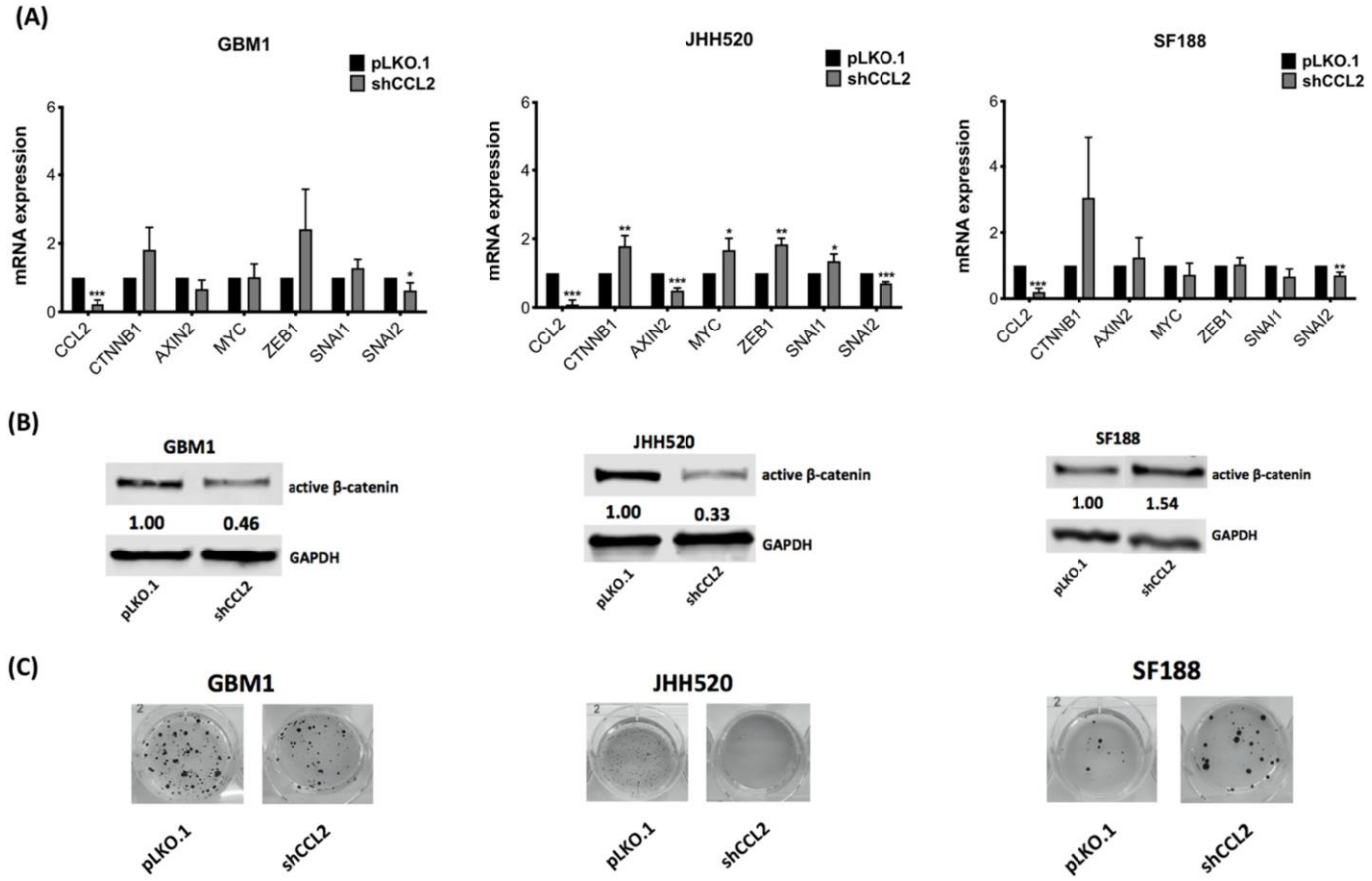

Figure 3. CCL2 knockdown alters expression of β-catenin target and related genes as well as clonogenicity of GBM cells: (**A**) β-catenin, the β-catenin target genes *Axin2*, *CCND1* and *c-Myc* and the β-catenin-associated genes *ZEB1*, *SNAI1* and *SNAI2* relative mRNA expression levels were analyzed by RT-qPCR in shCCL2 cells and compared to control cells (pLKO.1) (**B**) Non-phospho-(active)-β-catenin protein levels were detected using immunoblotting in shCCL2 and control cells (pLKO.1). (**C**) CCL2 suppression led to decreased clonogenicity of GBM1 and JHH520 while increasing clonogenicity of SF188 as detected by using a soft agar assay. Representative pictures of NBT stained colonies are shown. Abbreviations: NBT, 4-Nitro blue tetrazolium chloride. Data are presented as mean ± SD (n = 3). Statistical significance was calculated with unpaired *t*-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Next, we determined the clonogenic potential of CCL2 knockdown cells and found reduced colony-forming ability in GBM1 (not significant) and JHH520 ($p = 0.0043$), while the clonogenicity of SF188 was non-significantly elevated (Figures 3C and S4D). To determine the effect of CCL2 on β-catenin activity, we treated GBM cells with recombinant CCL2 and analyzed the expression levels of *CTNNB1* and *AXIN2*. We observed elevated *CTNNB1* levels in GBM1 cells ($p = 0.039$) and increased *AXIN2* levels in JHH520 ($p = 0.032$). In SF188, *AXIN2* expression levels were downregulated ($p = 0.0003$) (Supplementary Figure S5A). Western blot analysis confirmed that active β-catenin protein levels were significantly increased after CCL2 treatment in GBM1 ($p = 0.0064$) and JHH520 cells ($p < 0.0001$) (Supplementary Figure S5B).

3.4. The β-Catenin Inhibitor MSAB Reduces Viability, Active β-Catenin Levels, Clonogenicity and Expression of β-Catenin Associated Genes in GBM Cells

To add a pharmacological model to our study, we tested the effects of the β-catenin inhibitor MSAB on GBM cells. MSAB has been shown to bind to β-catenin protein leading to its degradation [65].

Thus, we treated GBM cell lines with MSAB and observed that it reduced cell viability in a dose-dependent manner (Figure 4A). Importantly, MSAB treatment also decreased active β-catenin protein levels in a dose-dependent manner (Figure 4B), confirming its β-catenin inhibitory effect. This was also evident in the soft agar clonogenicity assay, where a decrease in clonogenicity was observed after MSAB treatment (Figure 4C). Furthermore, treatment with MSAB for 24 h resulted in increased apoptosis of GBM cells (Figure 5B). To test whether the effect of MSAB was limited to β-catenin, we investigated the expression levels of its target genes using 10μM MSAB. Expression of *CTNNB1*, *AXIN2*, *MYC*, *ZEB1* and *SOX2* were significantly downregulated in all tested cell lines (Figure 4D). CCL2 expression was also downregulated in JHH520 cells (p = 0.0003), while we could not observe statistical significance in GBM1 and SF188. *SNAI1* and *SNAI2* were not altered by CCL2 knockdown in GBM1 and SF188.

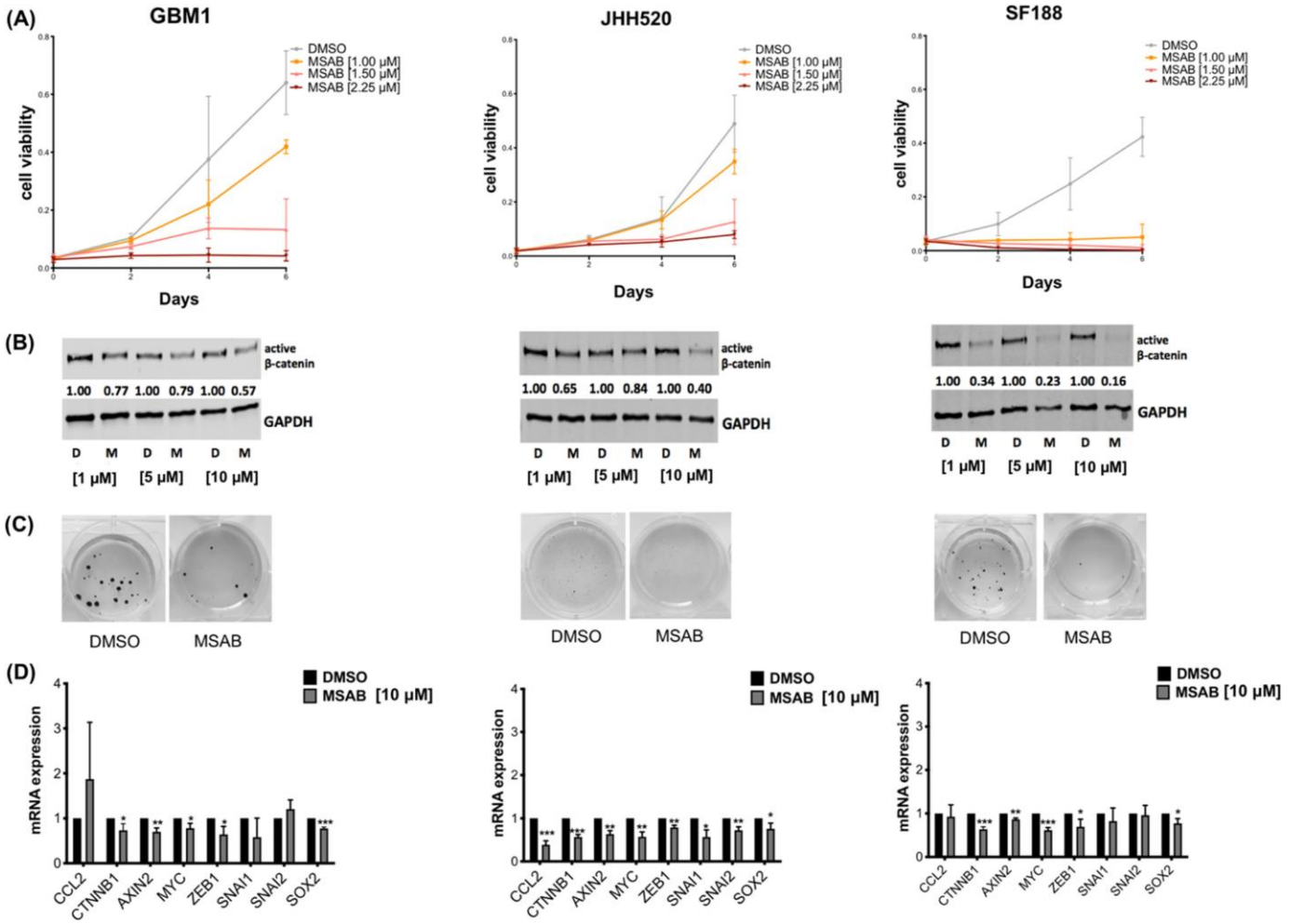

Figure 4. MSAB treatment reduces viability, active β-catenin protein levels and clonogenicity of GBM cells: (**A**) Cell viability was decreased by MSAB treatment in a dose-dependent manner. (**B**) Pharmacological β-catenin inhibition with MSAB led to suppression of non-phospho-(active) β-catenin in a dose-dependent manner as assessed by immunoblotting. Cells were treated with shown concentrations for 24 h. GAPDH was used as loading control. (**C**) MSAB treatment decreased clonogenicity of GBM cells in soft agar assay. Representative pictures of NBT stained colonies are shown. (**D**) The relative mRNA expression levels of β-catenin target genes (*Axin2*, *c-Myc*), -associated genes (*SNAI1*, *SNAI2*), neural stem cell marker *SOX2* and chemokine *CCL2* were measured by RT-qPCR in MSAB-treated cells compared to control cells (DMSO). Data are presented as mean ± SD (n = 3). Statistical significance was calculated with unpaired *t*-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

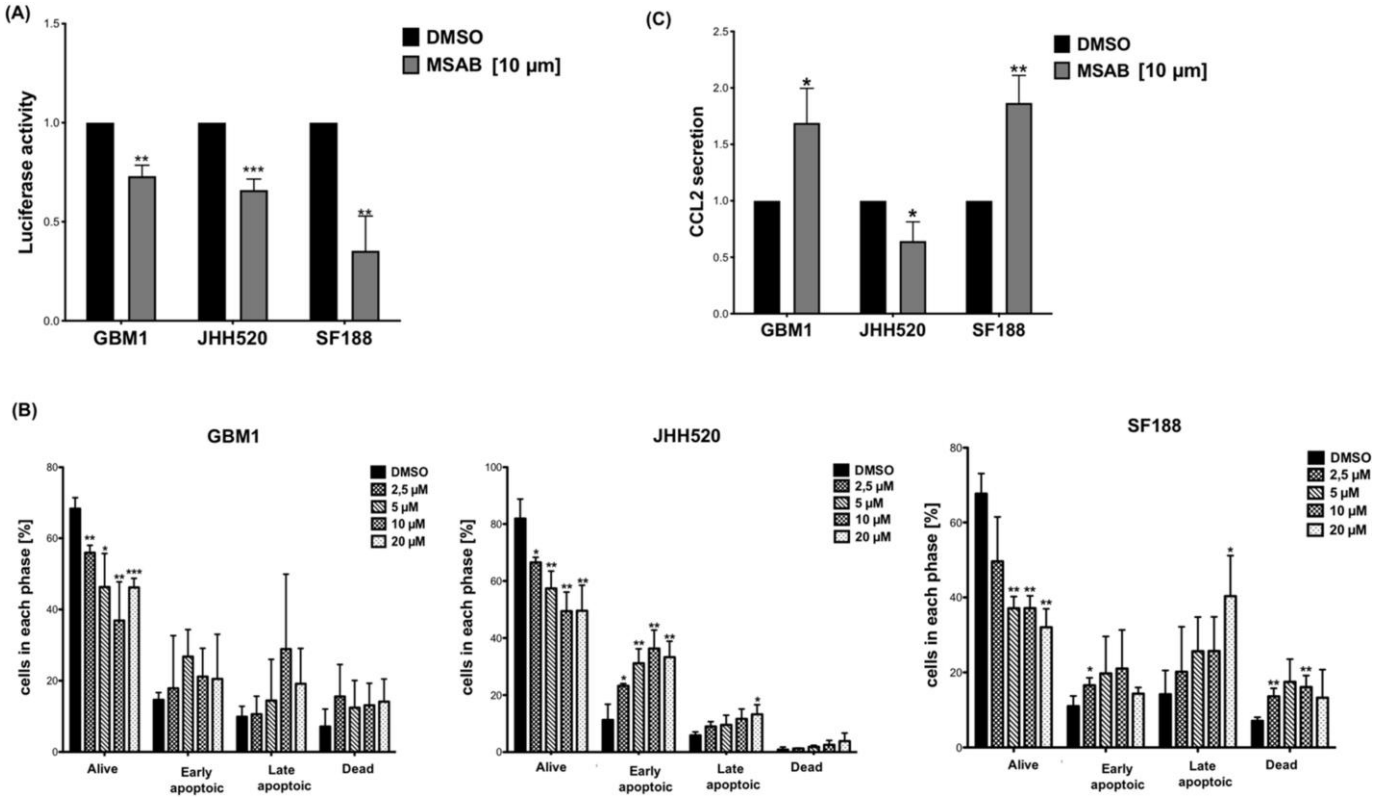

Figure 5. MSAB reduces Wnt-activity, induces apoptosis and modulates CCL2 secretion in GBM cells: (**A**) 24 h MSAB treatment (10 µM) reduced Wnt-activity in glioblastoma cell lines as assessed by Luciferase Reporter Assay. The relative luciferase activity data from three cell lines are shown. (**B**) 24 h treatment with MSAB induced apoptosis in GBM cell lines in a dose-dependent manner. Apoptosis was assessed with Muse Annexin V and Dead Cell Kit in three cell lines. (**C**) Altered CCL2 protein levels (relative CCL2 secretion) in the conditioned medium measured after 24 h incubation by ELISA. Data are presented as the mean ± SD (n = 3). Statistical significance was calculated with unpaired *t*-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.5. MSAB Decreases Wnt/β-Catenin-Activity and Modulates CCL2 Secretion

We used a Luciferase Reporter assay driven by CTNNB1/β-catenin binding to multimerized TCF/LEF promoter sites, to measure canonical Wnt/β-catenin activity in the cell lines. Wnt-signaling was significantly reduced in all three cell lines after treatment with MSAB (Figure 5A). Furthermore, treatment with MSAB for 24 h resulted in increased apoptosis of GBM cells (Figure 5B). Similarly, treatment of GBM cell lines with MSAB altered secretion of CCL2 levels (Figure 5C). Treatment of GBM cells with MSAB (24 h) increased CCL2 levels in the supernatants of GBM1 ($p = 0.0175$) and SF188 ($p = 0.0036$), while the supernatant of JHH520 ($p = 0.0224$) showed significantly reduced CCL2 levels (Figure 5C).

4. Discussion

In this study we showed that attraction of $CD14^+$-monocytes by GBM is reduced by genetically targeting β-catenin in vitro. RNA interference of both β-catenin and CCL2 in GBM cells reduced migration of $CD14^+$-monocytes towards TCM of glioblastoma cells. Furthermore, β-catenin knockdown decreased CCL2 secretion of glioblastoma cell lines, while CCL2 knockdown modulates β-catenin- and EMT-related genes. Pharmacological β-catenin inhibition with MSAB reduces Wnt/β-catenin activity and induces apoptosis in glioblastoma cells, while altering CCL2 secretion.

Therapy resistance and recurrence of GBM are associated with the presence of GSCs [5–10]. Previous studies have observed that β-catenin plays an important role in GBM, primarily

by promoting growth, invasion, and treatment resistance by maintaining the stem cell properties [19–28,76,77]. Since β-catenin is involved in immunological processes [29–33], we investigated the effect of β-catenin on attracting immune cells, in particular $CD14^+$-monocytes. GSCs were already associated with recruitment of tumor-supportive immune cells, such as TAMs and MDSCs, which derive from circulating monocytes [62,63,78,79]. TAMs in GBM have been shown to correlate with WHO grades [80] predicting the prognosis for high-grade glioma patients [81–83]. Interestingly, we observed that the treatment with TCM derived from β-catenin knockdown GBM cells reduced the migration of PBMCs and monocytes compared to control cells. Therefore, we hypothesize that β-catenin plays a key role in attracting precursor cells of TAMs/MDSCs to the tumor microenvironment.

In addition, we investigated whether CCL2 is involved in β-catenin-dependent cross talk between immune cells and GBM cells. Several studies emphasized the role of CCL2 in the GBM tumor microenvironment and in chemotaxis of tumor-supporting immune cells [47–49]. After β-catenin suppression we observed significantly reduced CCL2 levels in TCM of adult (GBM1 and JHH520) cell lines. The pediatric cell line SF188 showed a similar, but not significant effect. We added recombinant CCL2 to the TCM of shβ-catenin cells and observed that PBMC migration towards the TCM was restored. When comparing the effects of CCL2 and β-catenin knockdown on chemotaxis of monocytes, the two effects appear to be similar. These results suggest a pivotal involvement of CCL2 in β-catenin-stimulated PBMC attraction and in attraction of PBMC, in general. However, the exact mechanism of β-catenin-stimulated attraction of $CD14^+$-monocytes remains to be investigated. In the pediatric cell line SF188 CCL2, protein secretion was not significantly decreased in β-catenin knock-down cells, while a decrease in monocyte migration was observed, indicating additional mechanisms involved in β-catenin-dependent monocyte attraction. In several studies, CCL2 was also associated with tumor cell migration and metastasis [84]. In line with this, we observed that CCL2 affects β-catenin- and EMT-related gene expression. Our finding regarding the interdependence of β-catenin and CCL2 is supported by a recent study showing that the β-catenin-CCL2 feedback loop mediates crosstalk between breast cancer stem cells and macrophages [64]. In addition, CCR2—the most common receptor for CCL2 [72,85]—promotes stabilization and translocation of β-catenin via AKT/GSK3β signaling in colon cancer cells [73]. Therefore, further studies are required to assess these mechanisms and whether the β-catenin/CCL2 axis can be found in other types of cancers.

To investigate whether pharmacological targeting of β-catenin would recapitulate our findings obtained with genetically modified GBM cells, we used MSAB, which binds β-catenin and induces its degradation [65]. Consistent with previous studies, MSAB treatment reduced the expression level of Wnt-related genes and Wnt-signaling activity of glioblastoma cell lines. We observed that MSAB treatment reduced GBM cell viability/clonogenicity of GBM cells and induced apoptosis in a dose-dependent manner, confirming the effectiveness of pharmacological Wnt/β-catenin-inhibition in our model [22]. Surprisingly, pharmacological inhibition showed different effects on CCL2 secretion than genetic modulation. We hypothesize this could be due to a difference in the duration or potency of β-catenin suppression. MSAB-induced β-catenin suppression was weaker than genetical suppression (Figures 1B and 4B) and was performed over a shorter period. Further experiments with extended pharmacological suppression are required to verify these findings.

In this study we investigated the migration of $CD14^+$-monocytes (precursors of TAMs and MDSCs) towards TCM. It remains to be determined how β-catenin and CCL2 affect differentiated TAMs, MDSCs and other immune cells of the GBM microenvironment. Therefore, further co-culture experiments with direct tumor-immune cell interactions and in vivo approaches are warranted to support our observations.

In our experiments, we observed differences between the three GBM cell lines. It is worth mentioning that differences in adult and pediatric cell lines can be expected due to the inherent heterogeneity of cancer cell lines, genetic/epigenetic variability, and/or inter-individual differences, as previously discussed [86]. For our comprehensive gene

expression analysis, the effect of β-catenin knockdown on GBM cell lines showed clear differences. However, we found that multiple (though not completely overlapping) genes involved in the Wnt/β-catenin pathway were equally affected in all tested cell lines.

Furthermore, we saw discrepancies between mRNA and protein data: for example, SF188 showed increased mRNA levels of CCL2 (Supplementary S1B), but decreased protein levels (Figure 1B) after β-catenin knockdown (both not significant). We speculate this could be due to posttranslational or epigenetic changes as well as possible protein-to-transcription feedback [87]. Therefore, more GBM cell lines should be tested to determine whether the transcriptional subtype of GBM cells (classical, proneural and mesenchymal) influences the response to β-catenin inhibition, and whether the efficacy of MSAB can be enhanced by additional CCL2 suppression.

5. Conclusions

β-catenin and CCL2 are important determinants of monocyte attraction towards glioblastoma cells and show interdependence in vitro. Pharmacological β-catenin inhibition with MSAB decreases Wnt/β-catenin and leads to apoptosis in GBM cells.

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3 General Discussion and Conclusion

Immunotherapy in GBM has failed to significantly improve standard therapy due to impaired anti-tumor immunity [27,42]. High heterogeneity, low mutational burden, and immunosuppressive mechanisms are identified as disadvantageous conditions for an effective immune response against glioma cells [42]. To overcome cancer immunity there is a need to better understand the glioma immune microenvironment, which contains tumor-infiltrating immune cells and their interaction with the heterogeneous glioma subpopulations [57]. Wnt/β-catenin in GSCs and tumor-associated macrophages emerged as negative predictors for both anti-tumor immunity and prognosis [95,143,145,161]. Due to the immediate vicinity of GSCs and TAMs in glioblastoma tumor mass [135], this thesis investigates the influence of the Wnt/β-catenin pathway on the migration of $CD14^+$ monocytes, which are the major source of TAMs [88]. It is shown, that β-catenin in GSCs is involved in the recruitment of TAMs [1], thereby providing an intriguing therapeutic target to improve anti-tumor immunity and response to immunotherapy.

One part of this thesis addresses the therapeutic potential of targeting β-catenin in glioma stem cells: β-catenin in GBM cell lines was targeted genetically by using lentiviral vectors containing small hairpin RNA (shRNA) and pharmacologically by using small molecule inhibitor MSAB [1]. β-catenin is the central protein of the canonical Wnt pathway and is directly correlated to EMT [151], $CD8^+$ T cell exclusion [161], and resistance to immune checkpoint inhibition in various cancers including GBM [160,162]. Different from other Wnt/β-catenin dependent cancer entities mutations in genes encoding for Wnt pathway components like CTNNB1 and APC gene are rarely found in GBM [142]. However, it has been shown in several studies that Wnt/β-catenin is active in GBM – especially in the GSC subpopulation – due to epigenetic changes of Wnt pathway inhibitors [142,180,181]. In this study, glioblastoma cells were cultivated in neurospheres to enrich the stem cell portion [182]. Both genetical and pharmacological β-catenin suppression decreased the proliferation of glioblastoma cells [1]. The results confirmed previous literature identifying β-catenin as one driver of tumor growth in glioblastoma stem cells [150].

As part of this study small molecule inhibitor, MSAB was first tested in glioblastoma cells [1]. Hwang et al. already showed, that MSAB directly targets β-catenin and decreases viability and proliferation in Wnt/β-catenin dependent cancer cells, while there

was no effect in normal tissue cells or Wnt/β-catenin independent cancer cells [157]. Luciferase assay and measuring active β-catenin protein expression validated that Wnt/β-catenin signaling was successfully suppressed in tested cell lines [1]. As expected, MSAB decreased proliferation and increased apoptosis in all glioblastoma cell lines, verifying the Wnt/β-catenin dependency of glioblastoma stem cells [1]. Wnt/β-catenin target genes, especially the mRNA expression of oncogene MYC, EMT-activator ZEB1, and the stem cell marker SOX2, were decreased after pharmacological β-catenin suppression [1]. These three genes have previously been recognized as targets of the canonical Wnt/β-catenin pathway and inducers of both glioma growth and stem cell characteristics [117,151,183-185]. ZEB1 was identified as one of the key processors of epithelial-mesenchymal-like transition in GBM [117,151]. Concordant to this MSAB treatment resulted in a reduced clonogenicity as evaluated by soft agar assay [1].

Assessing the results of this thesis the therapeutic potential of β-catenin inhibition in the GSC subpopulation was underlined and MSAB was identified as a pharmacological candidate for further *in vivo* application. Pharmacological β-catenin inhibition in GBM was already performed in other preclinical studies: The compounds FH535, PKF115-584, and ICG-001 target the β-catenin transcription complex and could reduce glioma growth in preclinical studies [186-188]. In clinical studies canonical Wnt inhibitors were tested in other cancer entities [189]. Unfortunately, some of the trials were terminated due to the high occurrence of pathological fractures which can be explained by the significance of the canonical Wnt pathway in bone homeostasis [189]. However, PRI-724, a small molecule that disrupts β-catenin and its coactivator CREB binding protein (CBP), demonstrated clinical safety and is currently tested in combination with gemcitabine in pancreatic cancer patients [190,191]. At last, despite promising preclinical data β-catenin inhibition has not yet found its path into clinical testing in GBM. Here, it must be considered, that aberrant Wnt/β-catenin could only be found in a subset of glioblastoma patients and is mostly active in the intertumoral variable subpopulation of GSCs [142,145,150]. Still, Wnt/β-catenin in GSCs has a pharmacological relevance, because it influences the phenotype of GBM [102,108,109,143,150,192,193]. Performing pharmacological β-catenin inhibition should be considered in preselected patient groups with aberrant Wnt/β-catenin activation. Considering the predictable side effects on bone homeostasis and the possible impermeability of the BBB local drug delivery e.g. by intraventricular application could be performed. In GSCs Wnt/β-catenin signaling is extensively connected to other signaling cascades like Notch, Hedgehog,

RAS/RAF/MAPK, EGFR, and PI3k/Akt/mTOR [194-198]. To avoid therapy resistance due to reciprocal regulation mechanisms it may also be useful to combine anti-β-catenin therapy with other pharmacological compounds.

By targeting only β-catenin it must be mentioned, that the non-canonical Wnt pathway is not included in this thesis. However, non-canonical Wnt pathway and ligands like Wnt5a also contribute to the proliferation and invasion of GBM [154,155]. Especially in the mesenchymal subtype of GBM Wnt5a is highly expressed and part of the reason why the mesenchymal glioblastoma subtype acts more invasive [155]. Moreover, both canonical and non-canonical pathways have been associated with chemo- and radioresistance in GBM [112,155]. Here, a comparison to the effect of already tested pan Wnt inhibitors like LGK974 would be a possibility to further investigate the involvement of all parts of Wnt signaling.

The main part of this thesis focuses on the immunological effects of β-catenin, which showed extensive effects on cancer immunity [158]. This thesis aimed to investigate the influence of β-catenin on the migration of $CD14^+$ monocytes. The supernatant of shRNA-based β-catenin knockdown glioblastoma cells was collected and migration of $CD14^+$ monocytes was measured compared to control cells (pLKO.1) by Boyden chamber assay [1]. The analysis revealed, that after shRNA-based β-catenin suppression in GBM cells, migration of peripheral blood-derived mononuclear cells (PBMC) and $CD14^+$ monocytes is decreased [1]. Although the effect showed a clear tendency, the results were not statistically significant in all cell lines [1]. Here, PBMC donors' interindividual differences must be considered to explain variations between the single repetitions. $CD14^+$ monocytes are the main source for TAMs which are the dominant immune cell population in GBM and have been associated with a poorer prognosis in GBM [145]. In recent studies β-catenin was especially linked to the exclusion of $CD8^+$ T cells and impaired antigen presentation following a higher presence of regulatory T cells as well as dendritic cells with an immunosuppressive phenotype [161,165,199].

The results confirm the importance of β-catenin in cancer immunity and point out an additional role of β-catenin by revealing the momentousness in direct recruitment of $CD14^+$ monocyte cells towards GBM [1]. This is supported by the recent finding, that TAMs and GSCs are frequently found close to each other in GBM tumor mass [135]. Assessing the complexity of the GBM immune microenvironment with its heterogenous

cell subpopulations and reciprocal interaction [57], co-culturing, and *in vivo* models are needed to validate these observations. However, this thesis indicates that β-catenin could be a biomarker for TAM infiltration in GBM. It would therefore be interesting to see if β-catenin correlates with a higher presence of TAMs in patient samples. Since it could already have been shown that β-catenin and TAM infiltration both are unfavorable for immunotherapy [95,134,159,160], one can speculate if combination treatment of β-catenin suppression and immune checkpoint inhibition leads to a better therapy response in GBM. Interestingly, LGK974 is currently tested in cancer patients with advanced solid tumors in combination with the monoclonal PD-1 antibody spartalizumab [200].

After shRNA-based β-catenin knockdown, a significant decrease in mRNA expression and secretion of CC-chemokine ligand 2 (CCL2) in two cell lines was measured compared to control, indicating an involvement in β-catenin driven monocyte attraction [1]. Interestingly, by adding recombinant CCL2 to the supernatant of β-catenin knockdown cells the migration of PBMC was restored [1]. CCL2 or monocyte chemoattractant protein 1 (MCP-1) belongs to the C-C chemokine subfamily and is mainly involved in immune cell - especially monocyte- attraction. Preferentially, CCL2 binds to C-C chemokine receptor type 2 (CCR2) as the corresponding receptor [201]. Previous studies revealed, that CCL2 plays various roles in carcinogenesis [202]. It has been shown to stimulate tumor cell growth and proliferation inter alia by activating signaling pathways like PI3K/AKT [203] or MAPK/ERK [204]. CCL2/CCR2 is involved in extensive neovascularization of cancer cells and metastatic process [202]. CCL2 is also associated with the EMT process by activating transcription factors including Snail or Matrix metalloproteinases (MMPs) like MMP2 and MMP9 [205,206]. In the tumor immune microenvironment CCL2 recruits MDSCs, regulatory T cells, and TAMs to the tumor site, which all can support tumor growth and impair effector T cells [123]. In attendance of CCL2 monocytes preferably differentiate into M2-like phenotype, secreting CCL2 by themselves and creating a vicious cycle [82]. CCL2 expression is associated with lower overall survival in GBM patients, indicating the importance of this cytokine in glioma maintenance [123,207].

To further investigate the role of CCL2 in GBM, a genetic CCL2 knockdown was created in this thesis. A slight, but not significant decrease in proliferation of glioblastoma cells was measured, which partly confirmed an involvement in tumor growth [1]. Coherent to previous literature, the extent of impaired monocyte migration resembles the findings in

β-catenin knockdown GBM cells [1]. The correlation between CCL2 and TAMs is not a discovery in GBM: Previous literature already identified CCL2 to play a crucial role in recruitment towards and even the differentiation of TAMs and MDSCs within GBM tumor mass [123,208-212]. Recently, Zhenyi et al. showed, that EGFR and EGFRvIII cooperate to upregulate CCL2, resulting in macrophage infiltration of GBM [212]. The results in this thesis support, that CCL2 and β-catenin are crucial for GBM cells to recruit $CD14^+$ monocytes. Furthermore, CCL2 secretion reveals one possible mechanism for β-catenin-dependent monocyte attraction. To mention, despite showing a significant decrease of monocyte migration there is only a tendential decrease of CCL2 secretion in the analyzed pediatric cell line, indicating heterogenicity between the tested cell lines and additional underlying mechanisms [1]. Interestingly, in this cell line protein levels of both β-catenin and CCL2 were rather low compared to the other analyzed ones [1]. Furthermore, the CCL2 messenger-RNA (mRNA) level in this cell line was even non-significantly increased after β-catenin knockdown, which could imply β-catenin-independent feedback mechanisms to counteract CCL2 decrease [1]. In general, CCL2 seems to be involved in β-catenin-dependent monocyte recruitment [1], but further analysis of the secretome would be helpful to identify other involved components and mechanisms.

Surprisingly, CCL2 dynamics after genetic and pharmacological β-catenin suppression levels differed [1]. While in β-catenin knockdown glioblastoma cells all cell lines showed a significant or at least tendential decrease of CCL2 levels, in two out of three cell lines the CCL2 levels were significantly increased after MSAB treatment [1]. The divergent results in genetic and pharmacological models could be explained by the difference in β-catenin suppression stability. MSAB treatment has been performed for 24 hours, while stable shRNA knockdown was achieved after puromycin selection over six days [1]. Therefore, extensive pharmacological assays are needed to see, how the CCL2 secretion is developing over a longer treatment period. It is conceivable, that CCL2 secretion is increased as part of a rescue or feedback mechanism after a transient β-catenin suppression.

Interestingly, recent studies already showed a feedback loop between β-catenin and CCL2 in breast cancer cells [213,214]. Therefore, it was investigated which influence CCL2 knockdown in GBM has on β-catenin mRNA expression and protein level. Surprisingly, β-catenin protein levels in adult cell lines were decreased after CCL2 knockdown, while

the tested pediatric cell line showed an increase of β-catenin [1]. The clonogenic capacity of the cell lines was changed consistently with β-catenin protein levels [1]. Following, the question arose, if CCL2 can induce Wnt/β-catenin signaling. Indeed, treatment with recombinant CCL2 led to a higher protein level of active β-catenin in all glioma cell lines [1]. Moreover, mRNA expression level showed inconsistencies in both experiments, which suggests the presence of epigenetic posttranslational changes [1]. At last, while data show signs of interdependence between CCL2 and β-catenin in GBM, the detailed mechanism remains unclear. To note, Mestdagt et al. revealed CCL2 as a target of the β-catenin/TCF/LEF pathway in breast cancer cells [213]. Zhang et al. demonstrated, that β-catenin can either bind directly to the CCL2 promoter or indirectly via transcription factor 4 (TCF4) [214]. They showed a positive correlation between β-catenin, CCL2, and the infiltration of macrophages in breast cancer patients [214]. Inhibition of CCR2 and β-catenin even acted synergistically to decrease breast cancer growth [214]. Another recent study claims, that TAMs can activate β-catenin in breast cancer stem cells by secretion of CCL2 [215]. TAM-derived CCL2 increased β-catenin expression and enhanced its nuclear accumulation by inducing Akt activity [215].

Further work is needed to transfer these findings to GBM, as CCL2 and β-catenin could act both as diagnostic markers of immunogenicity of GBM and therapeutic targets. Pharmacological CCL2 inhibition was already performed in preclinical studies: the monoclonal antibody C1142 which can specifically neutralize CCL2 significantly reduced TAM and MDSC infiltration in gliomas *in vivo* and successfully inhibited tumor growth, followed by a prolonged survival [209]. Another CCL2 inhibitor called mNOX-36 delivered promising results in gliomas in combination with the already used VEGF inhibitor Bevacizumab *in vitro* [216]. Since the CCL2-CCR2 axis is involved in tumor growth, invasion, and cancer immunity, it could be sensible to directly combine it with immune checkpoint inhibition [202]. For example, a CCR2 inhibitor improved the effect of immune checkpoint inhibition in murine tumors [217]. As CCL2 is an important cytokine in immunological processes, local drug application could be needed to avoid systemic side effects. Moreover, in a pharmacological study with a CCL2 antibody, it was investigated, that only transient CCL2 suppression led to a subsequent increase to 1000-fold higher concentrations and even more enhanced metastasis [218,219]. Therefore, pharmacological CCL2 suppression needs to be performed efficiently and continuously, which could be challenging [218,219]. Based on the results of this thesis, one could speculate that a combination treatment including pharmacological

CCL2/CCR2 and β-catenin inhibition could reduce TAM infiltration and improve immune checkpoint inhibition in glioma.

In conclusion, this thesis reveals, that β-catenin is a major player in the recruitment of $CD14^+$ monocytes towards glioma stem cells *in vitro.* It further confirms the predominant role of CCL2 in this process and implicates an interdependence between β-catenin and CCL2 *in vitro*. Based on these results both β-catenin and CCL2 are potential targets to improve anti-tumor immunity – especially in combination with immunotherapy like immune checkpoint inhibitors. *In vivo* studies in xenografts need to confirm these observations. Following, clinical studies with pharmacological inhibition of the Wnt/β-catenin pathway and/or CCL2-CCR2 pathway in combination with checkpoint inhibition would be a logical advancement.

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Supplementary figures:

Crosstalk between β-catenin and CCL2 drives migration of monocytes towards glioblastoma cells

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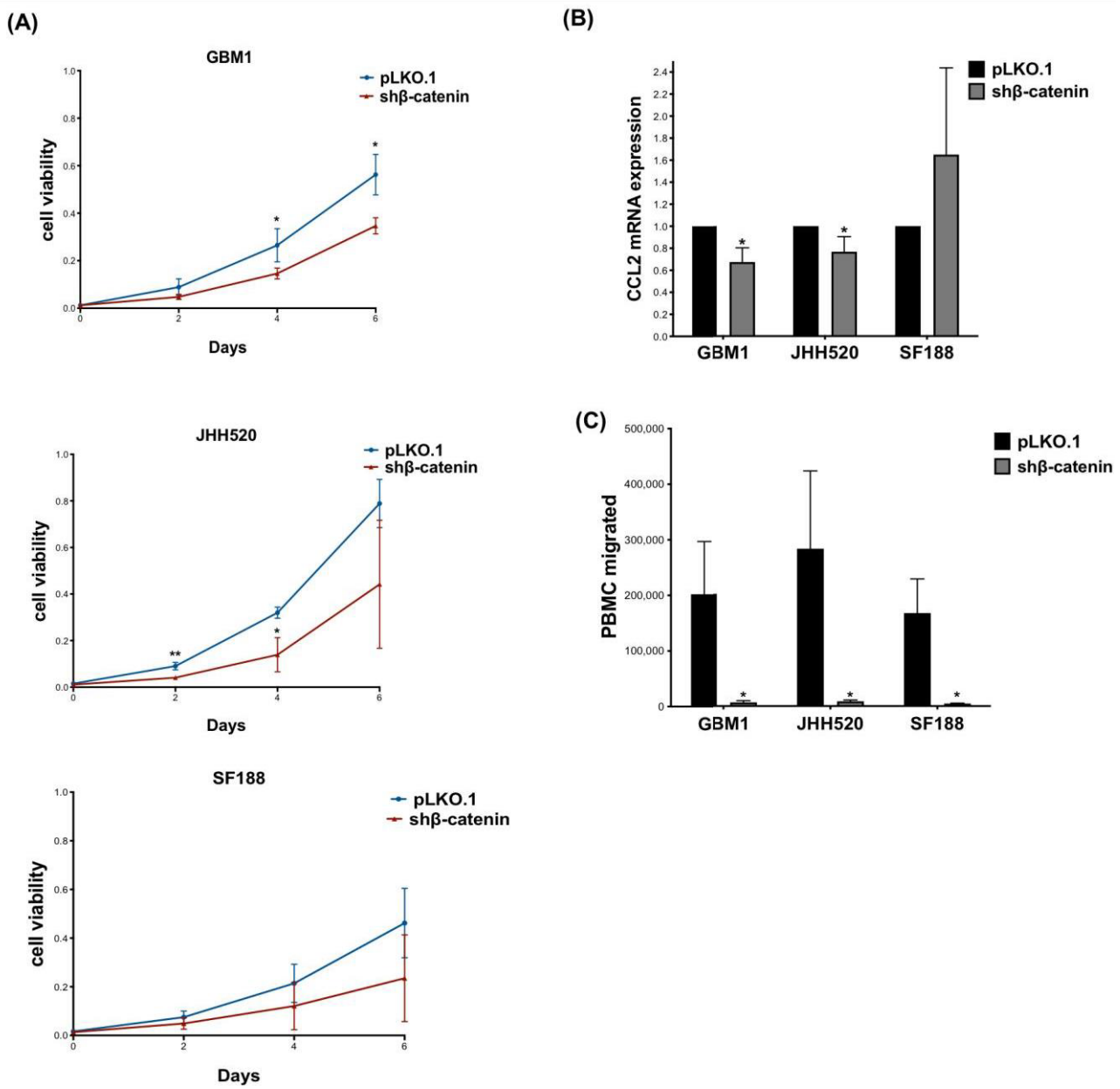
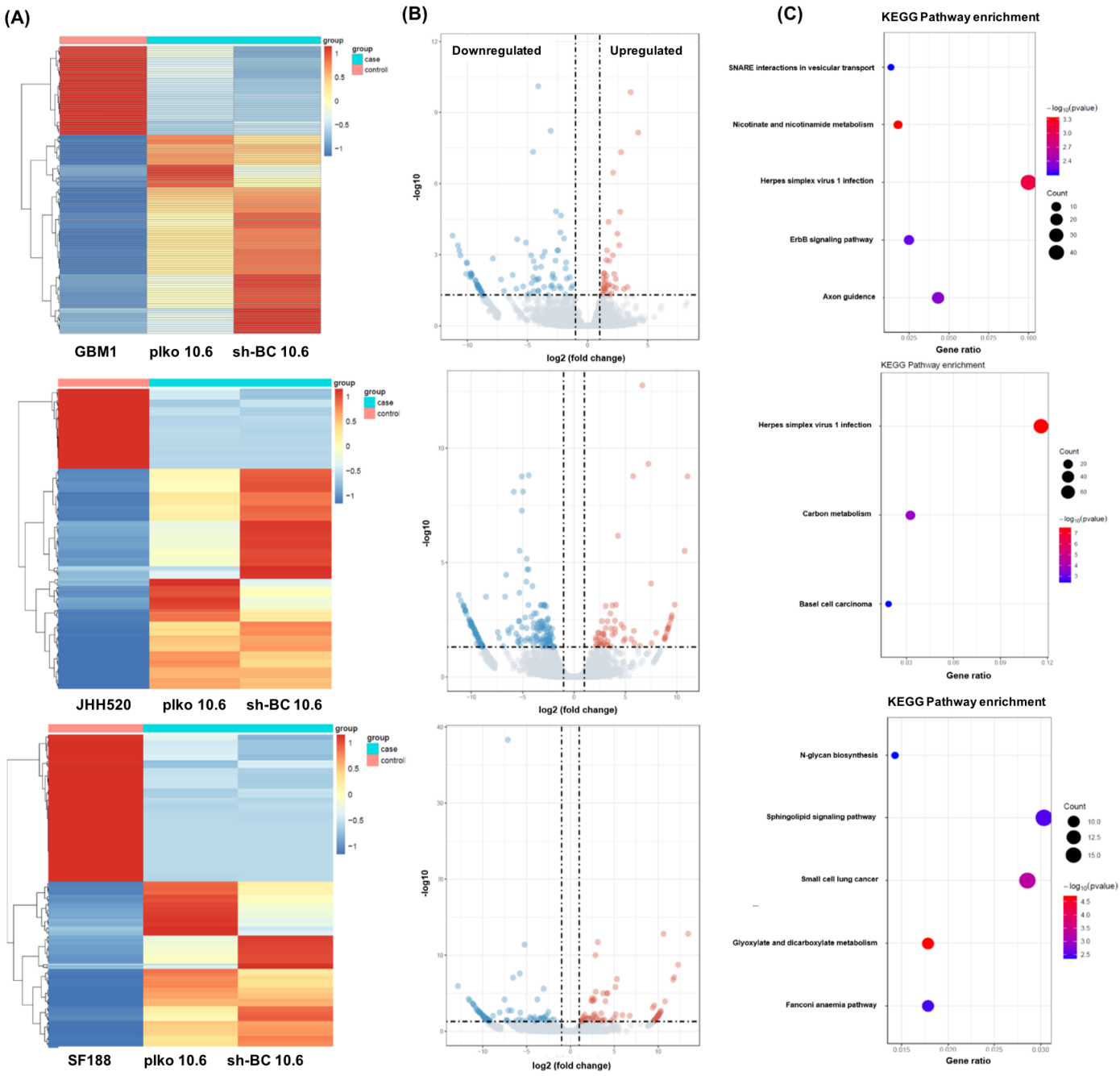
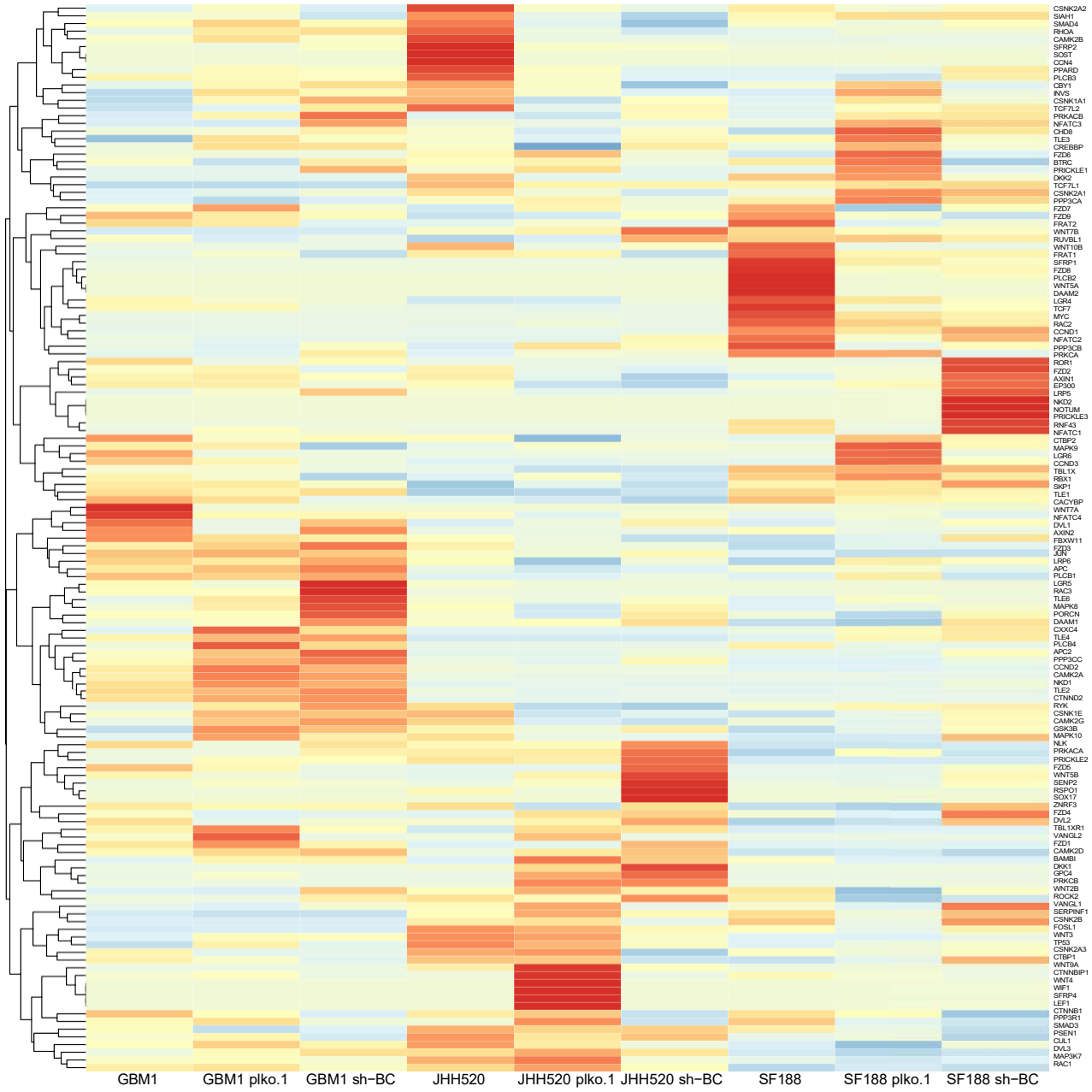


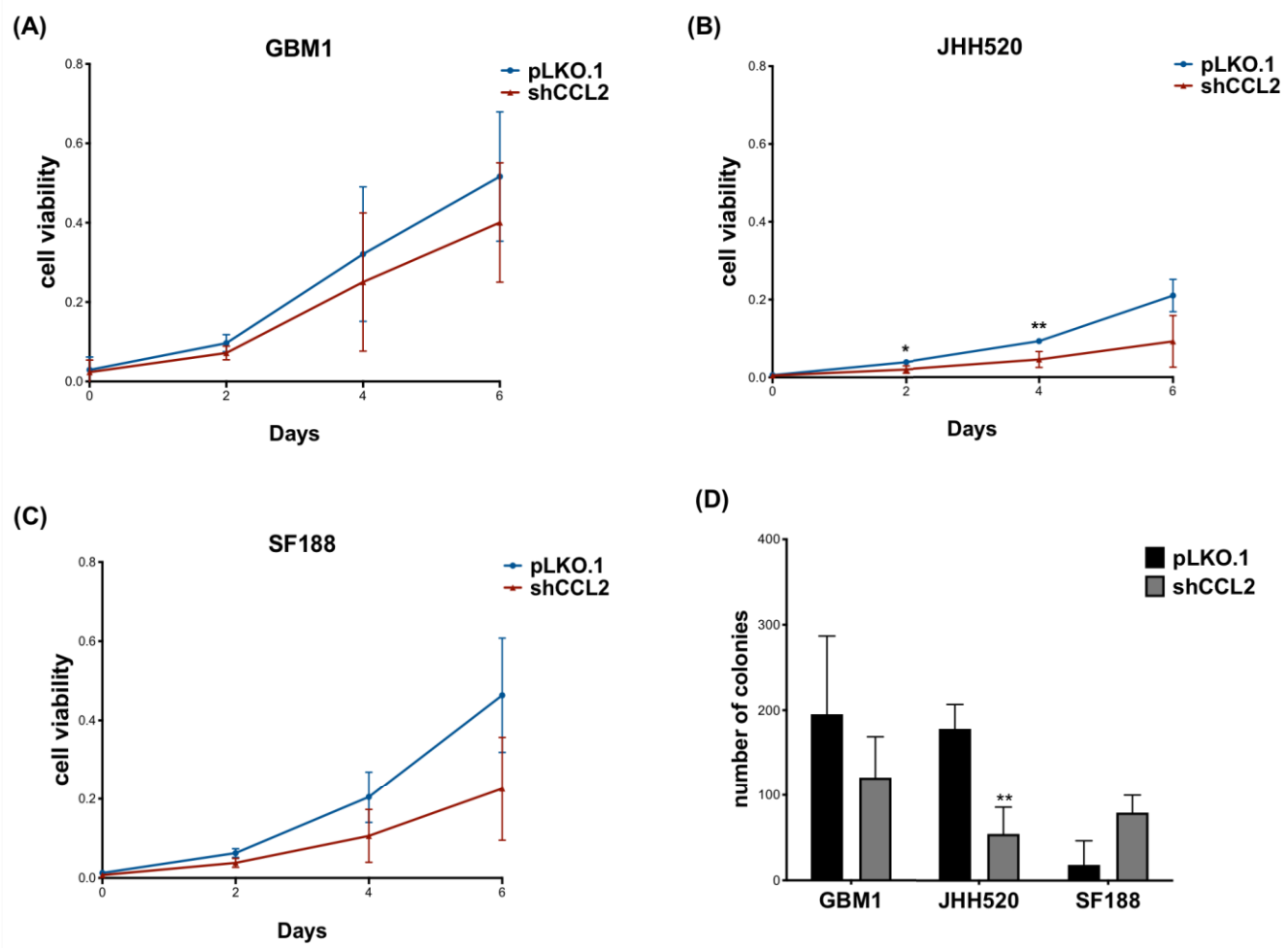
Supplementary Figure S1: β-catenin knockdown partially decreases cell viability and CCL2 mRNA expression in GBM cells. GBM cell lines (GBM1, JHH520, SF188) were transduced with lentiviral particles containing shβ-catenin plasmids: **(A)** Cell viability was partially reduced compared to control (pLKO.1) cells. Exponential growth curves were calculated for each condition. **(B)** The relative CCL2 mRNA expression levels were analysed by RT-qPCR in shβ-catenin cells and compared to controls (pLKO.1). **(C)** PBMC migration towards TCM of shβ-catenin GBM cells was significantly decreased compared to control (pLKO.1). Data are presented as mean ± SD (n=3). Statistical significance was calculated with unpaired t-test. * p ≤0.05 **p ≤0.01 ***p ≤0.001.



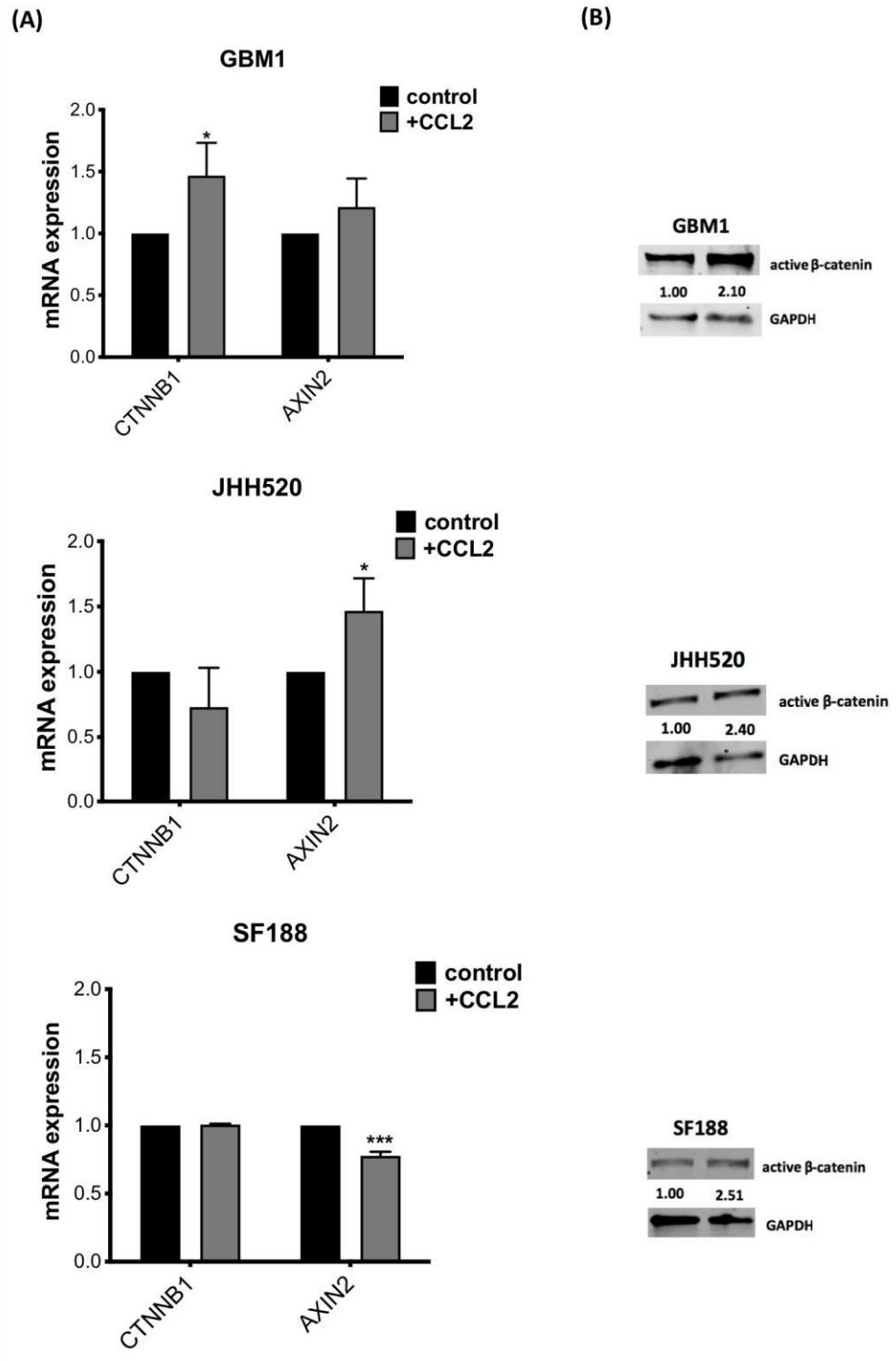
Supplementary Figure S2: Genome-wide gene expression analysis of three GBM cell lines: **(A)** heatmap showing variation of gene expression in control (pLKO.1) cells and shβ-catenin cells, **(B)** altered genes compared to the control cells (plKO.1), **(C)** KEGG associated pathways.



Supplementary Figure S3: A heat map shows changes in expression of genes involved in the Wnt/β-catenin pathway in three cell lines, control (pLKO.1) cells and shβ-catenin cells.



Supplementary Figure S4: CCL2 mediated cell viability and clonogenicity of GBM cells:
(A)–(C) Exponential growth curves representing cell viability in cell lines compared to control (pLKO.1) cells measured by MTT assay. **(D)** CCL2 suppression led to decreased clonogenicity of GBM1 and JHH520 while increasing clonogenicity of SF188 as detected by using a soft agar assay. Quantifications of three colony forming assays are shown. Data are presented as mean ± SD (n=3). Statistical significance was calculated with unpaired t-test. * p ≤0.05 **p ≤0.01 ***p≤ 0.001.



Supplementary Figure S5: Treatment with recombinant CCL2 alters β-catenin -activity of GBM cells: (**A**) The relative mRNA expression levels of β-catenin gene (*CTNNB1*) and target gene *AXIN2* were measured by RT-qPCR in CCL2-treated cells compared to control cells (PBS/BSA 0,1%). Cells were treated with 100 ng/ml recombinant CCL2 for 30 minutes. (**B**) Non-phospho- (active) β-catenin protein levels were increased after CCL2 treatment as assessed by immunoblotting. Data are presented as mean ± SD (n=3). Statistical significance was calculated with unpaired t-test. * $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.001$.

Supplementary Table S1: Primer sequences used in RT qPCR

Primer	forward	reverse
β-actin	CCCAGCACAATGAAGATCAA	CGATCCACACGGAGTACTTG
CTNNB1	GGGCCTCAGAGAGCTGAGTA	TGAGCAGCATCAAACTGTGTAG
AXIN2	AGCCAAAGCGATCTACAAAAGG	GGTAGGCATTTTCCTCCATCAC
c-MYC	CCTTAATTAAAATGCCCCTCAACGTTAGCT	GGAATTCCATATGTTACGCACAAGAGTTCCGTA
SNAI1	GCTGCAGGACTCTAATCC	ATCTCCGGAGGTGGGATC
SNAI2	TGGTTGCTTCAAGGACACAT	GTTGCAGTGAGGGCAAGAA
ZEB1	AAGAATTCACAGTGGAG AGAAGCCA	CGTTTCTTGCAGTTTGGGCATT
SOX2	TGGACAGTTACGCGCACA	CGAGTAGGACATGCTGTA
CCL2	AGGTGACTGGGGCATTGAT	GCCTCCAGCATGAAAGTCTC

6 Danksagung

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